THYMOXYETHYL-DIETHYLAMINE ANTAGONISM AND ANTI-HISTAMINES[1]

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I. THYMOXYETHYL-DIETHYLAMINE ANTAGONISM.

Owing to the extreme toxicity of 929F (thymoxyethyl-diethylamine), it was thought worth while to investigate the possibility of detecting a suitable therapeutic antagonist for this compound which might inhibit the severe cardiovascular effect. A report appeared by Okazaki (1932a) who submitted evidence that adrenalin was an antagonist for phenoxyethylalkylamines. However, a perusal of his tracings shows that in some cases the blood pressure was already on the recovery stage when adrenalin was injected and, though the blood pressure probably returned to normal more rapidly, antagonism such as that of atropine for acetylcholine was not demonstrated. With gravitol and tastromin the antagonism was more notable, but could easily be nonspecific. Further, with toxic doses of the thymoxy type compounds, central motor paralytic phenomena are observed (Okazaki, 1932b). It was thought worth while to determine if there were any antagonism some minutes after injection of adrenalin when the blood pressure had returned to normal.

METHOD.

The carotid blood pressure was recorded in cats under artificial respiration and chloralose anaesthesia after preliminary induction with ether, and in one instance a little chloroform in addition. In one cat the vagi were cut, and in the remaining two, the vagi were left intact. All injections were made into the femoral vein.

EXPERIMENTAL.

The findings are shown in Fig. 1 and Fig. 2 A and B.

In Fig. 1, the blood pressure record is shown of a cat weighing 3·5 kg. in which the vagi were cut. The depression due to 1 mg. of crude 929F (at T) is seen to be approximately equal to that of 1 ml. of 1 in 2 million histamine (at H), i.e. 0·5γ (first panel). Between the first and second panels, 2 mg. of atropine were injected twice intravenously and the interval between these panels was 15 minutes. At T (second panel) the response to 1 mg. 929F was unchanged 20 minutes after the initial injection of atropine. Doses of 1 mg. 929F given five minutes before and five minutes after this dose (not shown) were similarly equal. Thirty minutes after the initial injection of atropine (T third panel), the response to 1 mg. 929F was little unchanged, and at H (third panel) three minutes later, it is seen that the depressant response to 1 ml. 1 in 2 million histamine was abolished, a very slight rise only occurring. Between the third and fourth panels 0·1 ml. of 1 in 1,000 adrenalin was injected, the blood pressure increased by 160 mm. Hg., and at T (fourth panel) 4 minutes later immediately the blood pressure had returned to normal it is seen that the response to 1 mg. 929F was reduced by about half.

In Fig. 2 A and B are shown the carotid pressure records from two cats (2·5 kg.) in both of which the vagi were intact. No atropine was injected.

In Fig. 2A at T is shown the response to 1 mg. 929F injected intravenously. Between the second and third panels 1 ml. of 1 in 1,000 neosynephrin was injected, and, when the blood pressure had returned to normal, after a rise of 120 mm. Hg. (in 5 minutes), 1 mg. 929F was

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again injected (T third panel). There was no diminution in the response. Between the third and fourth panels 0·1 ml. adrenalin 1 in 1,000 was injected the blood pressure rose 110 mm. Hg., and 2 minutes 45 seconds later when the pressure had returned to normal, 1 mg. 929F gave a slightly diminished response (T fourth panel).

In Fig. 2B are shown the vascular responses in another cat. At T, 8 mg. thymoxyethyldiethylamine were injected, and at E, 1 ml. of 1 in 100 ephedrine followed by a rise in pressure of 55 mg. Hg. The rise in pressure is long maintained following administration of ephedrine, and it is seen that 15 minutes after the injection the response to 8 mg. 929F (at T) was increased. This may be related to the initial high blood pressure.

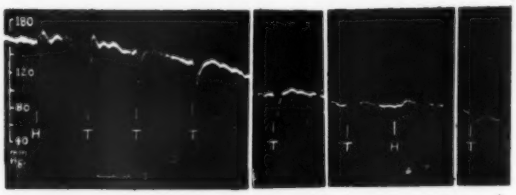

Fig. 1. Cat Blood Pressure. Time in minutes. Details in text.

II. ANTI-HISTAMINES.

Various analogues have been tried in an endeavour to obtain a less toxic antihistamine than thymoxyethyldiethylamine. They are: Thymoxyethyldiethylamine hydrochloride (1), N Diethyl N^1 ethylphenyl ethylenediamine hydrochloride (2), N Diethyl N^1 ethylphenyl ethylenediamine benzoate (3), Carvacryl-diethylamino ethyl sulphide hydrochloride (4), 3:6-Di (diethylaminoethoxy) p-cyeme sulphate (5), Purified thymoxyethyldiethylamine hydrochloride (6). These compounds were made in the laboratory of Professor Trikojus for the C.D.B. Australia.

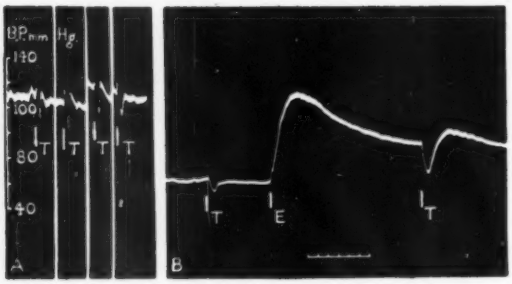

Fig. 2. Cat Blood Pressure. Time in minutes Details in text.

METHOD.

Tests were made of the ability of depressing the response of the guinea-pig jejunum to histamine. Assessment was made by bracketing the effect of a selected dose on a standard quantity of histamine (0·06γ) with a sample of thymoxyethyldiethylamine prepared by Dr. Fantl, of the Baker Institute, Melbourne. The dose was selected so that inhibition was not complete when it was added to the bath. The time taken for the response to histamine after even washings-out to return to normal was recorded. These procedures allowed assessment.

EXPERIMENTAL.

The findings are recorded in Table 1.

TABLE 1.

Anti-histamine activity.

Sample 1. 28 p.c. $l.d._{50}$ approximately 1,630 mg. per kg. (mice).
Sample 2. 4 p.c.—unstable.
Sample 3. Negligible.
Sample 4. 12 p.c.
Sample 5. Negligible.
Sample 6. 100 p.c.

It is noted that the first sample of thymoxyethyldiethylamine had only 28 p.c. of the activity of the Fantl sample with an increased $l.d._{50}$ (1,630 mg./kg., mice): when purified (6) the activity rose to 100 p.c.

Since none of the other samples was found to have potent activity (Table 1) (compare thymoxyethyldiethylamine) no further work was done with these compounds.

SUMMARY.

Adrenalin 0·1 ml. of 1 in 1,000 has a slight antagonism for the succeeding vascular depressant effect of thymoxyethyldiethylamine in doses of 1 mg. Atropine, ephedrine and neosynephrine do not appear to exhibit such antagonism.

Thymoxyethyldiethylamine analogues tested were relatively inactive as anti-histamines.

REFERENCES.

Okazaki (1932a): Jap. J. med. Sci., Pharmacology 6, p. 23.
Okazaki (1932b): *Ibid.*, 6, p. 133.

TREATMENT OF PHOSGENE POISONING WITH THYMOXYETHYLDIETHYLAMINE[1]

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Following the work reported earlier (Trethewie, 1947), an attempt has been made to reproduce in dogs and mice the favourable effect of the use of thymoxyethyldiethylamine in guinea-pigs following exposure to phosgene.

The dog lung, like that of the guinea-pig and man, is rich in histamine, and it was, therefore, thought desirable to determine if thymoxyethyl β diethylamine, in spite of toxicity, could be used to lower the mortality of dogs following exposure to phosgene, as is the case in the guinea-pig. Mice were treated similarly.

I. DOGS.

Method.

Phosgene concentrations were determined in a 30 metre chamber by F. R. Gell (M.S.L.) by the chloride method on samples obtained at the beginning and end of exposure. Dogs were introduced four at a time, and exposed to concentrations of 161-268 mg./c.m. for periods of 19-35 minutes. The c.t. (mg./cubic metre $\times$ time minutes) range was from 3,050–6,230. Dogs were selected in pairs of approximately similar breed and weight, and subcutaneous injections of thymoxyethyl β diethylamine were decided by cards at random. Thirty dogs were used. Distilled water was injected into the controls. Two dogs that were not exposed to phosgene received injections of thymoxyethyldiethylamine only. Post-mortem examination was performed on all animals, survivals being killed after 19–28 days.

Result.

The toxicity of thymoxyethyldiethylamine for dogs could not be readily determined since only a limited amount of it was available. Staub (1939) states that 5 mg. per kg. causes vomiting in this animal. In this experiment, two dogs, weighing 7·7 and 8·2 kg. (Nos. 1 and 2 respectively), were injected subcutaneously with 10 mg. thymoxyethyldiethylamine HCl. The results are recorded in Table 1. These findings confirmed those of Staub and suggested that it would not be possible to give doses comparable to those given to guinea-pigs in the earlier work where this treatment was satisfactory after exposure to phosgene.

TABLE 1.

	Dog 1.	Dog 2.
00 hours	Injected 10 mg./kg. s.c.	Injected 10 mg./kg.
8 minutes	Excited—howled and barked	Excited—howled and barked
36 minutes	Vomited	
01 hours	Well	Well
20 hours	Injected 20 mg./kg.	
21 hours		"Cold," did not seem well
23½ hours	Huddled in kennel	
24½ hours	Vomited	
48 hours	Quiet	
72 hours	Well	Well

It was thought that, since histamine did not appear in the lung perfusion fluid for 5–6 hours after one minute's exposure to phosgene and 80 mg./kg. thymoxyethyldiethylamine 15 minutes after exposure was necessary to lower the mortality in guinea-pigs (Trethewie, 1947), that 10 mg./kg. doses injected immediately after exposure and again 4–5 and 8–9 hours later

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might be satisfactory in dogs in spite of the evident toxicity. Such was not the case. The mortality was significantly higher in the treated than in the untreated animals, the probability of chance explaining the result being 0·0206 (Table 2).

TABLE 2.

Dose.	c.t.	Deaths. Treated.	Untreated.
30 mg./kg.	3,050	1/2	0/2
30 mg./kg.	3,900	2/2	0/2
30 mg./kg.	3,500	1/2	0/2
30 mg./kg.	5,400	1/2	1/2
30 mg./kg.	5,460	2/2	0/2
30 mg./kg.	6,200	0/2	0/2
5 mg./kg.	6,230*	1/1	1/1

* There was only one dog in each group in this experiment since one untreated dog escaped, and, therefore, the paired treated dog which survived had to be rejected.

If one includes only the doses 30 mg./kg. the probability of the findings (7/12 deaths (treated) and 1/12 deaths (untreated)) being due to chance is less still, namely, 0·0136.

Evidently the drug is too toxic for dogs in this dose.

A comparison of effectiveness of the drug to c.t. phosgene in the dog and guinea-pig is given in the two Tables which show, in Table 3, survivals including the times of injection, and in Table 4 the ratio of drug to c.t. exposure with survivals.

TABLE 3.

Exposures.	Animal.	Dose thymoxy salt.	Survivals. Treated.	Untreated.
1,500–2,300	Guinea-pig	80 mg./kg. 15 mins. after exposure	10/16	2/16
5,715	Guinea-pig	40 mg./kg. 30 mins. after exposure 40 mg./kg. 2½ hrs. after exposure	0/4	0/4
4,590	Guinea-pig	80 mg./kg. 12 mins. before exposure	0/4	1/4
3,050–6,200	Dog	10 mg./kg. 25–40 mins. after exposure 10 mg./kg. 4–4½ hours after exposure 10 mg./kg. 7–9½ hours after exposure	5/12	11/12
6,230	Dog	5 mg./kg. 40 mins. after exposure	0/1	0/1

TABLE 4.

Animal	$\frac{\text{Dose thymoxy salt (mg./kg.)}}{\text{Exposure phosgene (mg./m}^3 \times \text{t in minutes)}} \times 1{,}000$.	Survivals. Treated.	Untreated.
Guinea-pig	35–53	10/16	2/16
Guinea-pig	14–17	0/8	1/8
Dog	5–10	5/12	11/12
Dog	1	0/1	0/1

Since in the guinea-pig a range of drug to c.t. × 1,000 of 35–53 is effective but a range of 14–17 is ineffective in the treatment of phosgene exposure, it is not surprising that a range of 5–10 is also ineffective with dogs.

Discussion.

It has proved impossible as yet to reproduce in dogs, the extremely favourable results obtained in the guinea-pig treated by this method. Preliminary results suggest that this discrepancy is due to the much greater toxicity of the drug for dogs. It does not seem that any modification in the doses would produce significant benefit, as the minimum dose required for satisfactory therapeutic effect is already sufficiently great to be extremely toxic. It is considered that it might be possible to obtain a drug which, while having a sufficiently low toxicity for dogs (and man), would still retain its therapeutic value in phosgene poisoning.

II. MICE.

Method

The mice were exposed to phosgene in pairs in four compartments in two cages for 11–15¼ minutes. Fourteen to thirty-one minutes after the beginning of exposure thymoxyethyldiethylamine was injected subcutaneously in a concentration of 100 mg./ml. in saline. Survivals were killed after 15–18 days.

Result.

Staub (1939) states that while the LD_{50} for mice is 500 mg./kg. a dose of 50 mg./kg. is greater than the tolerance dose. With doses of 88 mg./kg. and upwards this finding has been confirmed for this sample. The mice lie flattened and extended, dyspnoea is noticed and they do not recover for some hours. A pair of mice receiving 650 mg./kg. of the drug subcutaneously survived and a pair receiving 1,000 mg./kg. both died.

It would appear that the aniline derivative 1571F would be more suitable for mice since 100 mg./kg. is well tolerated. This was not available.

TABLE 5.

Duration of exposure (min.)	Phosgene concn. mg./m³ × min.	Time in mins. injected after exposure.	Dose drug mg./kg. (S.C.)	Mortality. Day of exposure. Treated.	Day of exposure. Untreated.	Total mortality. Treated.	Total mortality. Untreated.	R*
12	2,880	15–18	88	0/8	3/8	7/8	7/8	28
10	2,065	14–20	100	—	—	1/8	2/8	43
15¼	2,944	14–31	110	1/8	—	5/8	4/8	34
11	2,381	14–20	145	—	—	4/8	1/8	53

R*. Ratio drug dosage mg./kg. (allowing 90 p.c. purity) to phosgene exposure mg./m³ × t multiplied by 1,000.

The findings in phosgenized mice are shown in Table 5. It is noted that 17 out of 32 of the treated mice died and 14 out of 32 of the untreated died. Thus there is no reduction in the mortality of phosgene poisoning in mice following treatment with thymoxyethyldiethylamine and possibly this is due to the drug's toxicity.

SUMMARY.

The mortality of dogs exposed to phosgene (c.t. 3,050–6,200) was increased following the injection of 30 mg./kg. thymoxyethyldiethylamine.

There was no reduction in the mortality of mice when treated with 88–100 mg./kg. thymoxyethyldiethylamine after exposure to phosgene (c.t. 2,065–2,944).

The significance of these findings in relation to the favourable effect of such treatment in guinea-pigs is discussed.

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Trethewie, E. R. (1947): Med. J. Austral., 1, p. 612.

STUDIES IN INFECTIOUS ECTROMELIA IN MICE (MOUSE-POX)

III. NATURAL TRANSMISSION: ELIMINATION OF THE VIRUS.

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In an earlier paper (Fenner, 1947b) existing information on the natural transmission of ectromelia was reviewed, and it was established that under natural conditions infection usually occurred by entry of the virus through a minute abrasion in the skin of the host. In this paper an attempt is made to define the important paths of elimination of the virus and the duration of infectivity of infected mice.

MATERIAL AND METHODS.

The material and methods used for the production and titration of stock virus, and for the estimation of the antibody response in infected mice, have been described in detail previously (Fenner, 1947a).

The "Hampstead" and the "Moscow" strains of ectromelia were used in different experiments, stock virus being obtained from infected chorioallantoic membranes.

Various discharges or washings from various areas were collected in a mixture of equal parts of 5 p.c. sodium sulphadiazine and penicillin (1,000 units per ml.). This inhibited bacterial growth and allowed satisfactory titrations for ectromelia to be made on the chorioallantois. Faecal suspensions, after preliminary centrifugation, were treated for five minutes with one-third of their volume of ether.

Materials were tested by two methods which were usually combined. Undiluted suspensions of the material, which often caused nonspecific lesions on the chorioallantois, were tested for ectromelia virus by pad inoculation of two mice. The development of a local lesion, the lesions of the internal organs and the serum ectromelia anti-haemagglutinin (E-AHA) titre at the end of fourteen days, were used as criteria of specific infection. The concentration of virus was determined by the inoculation of serial tenfold dilutions of the material being tested on the chorioallantois of twelve-day-old chick embryos.

For histological purposes organs were fixed in formol-saline and stained with haematoxylin and eosin or with Mann's stain.

EXPERIMENTAL RESULTS.

In an attempt to define the relative importance of different possible routes of elimination of ectromelia virus two preliminary studies were carried out, firstly a determination of the concentration of virus in various organs at intervals after inoculation of a suspension into the tissues of the tail, and secondly an investigation of the duration of infectivity of infected mice.

SPREAD OF VIRUS THROUGH THE ORGANS OF INFECTED MICE.

Forty-five eight-weeks-old male mice were inoculated in the tissues of the tail with 0·05 ml. "Hampstead" ectromelia virus, diluted 1:10.

At intervals after the inoculation batches of three mice were killed by bleeding from the axilla under anaesthesia. The blood was either defibrinated and the plasma and washed cells tested for virus, or allowed to clot and the serum used for antibody tests. The organs from one mouse were removed and fixed in formol-saline for subsequent histological examination. From the other two mice the organs were removed aseptically, ground with sand, taken up in the

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volume indicated of sterile, normal horse-serum-saline (NHSS) and 0·05 ml. of the supernatant after centrifugation inoculated in various dilutions on the chorioallantois of twelve-day-old eggs. The membranes were removed after three days' incubation at 35° C. and examined under a binocular dissecting microscope. The organs treated thus were: nasal scrapings, removed with a curette and taken up in 0·5 ml. NHSS, the inguinal lymph nodes (0·5 ml. NHSS), the kidneys (1·0 ml. NHSS), the lungs (1·0 ml. NHSS), the spleen (1·0 ml. NHSS), a portion of liver the size of the spleen (1·0 ml. NHSS) and the left testis (1·0 ml. NHSS). The tail, the small intestine and its contents, freed as much as possible from the mesentery, and the contents of the large intestine, were ground with sand in a mortar and each taken up in 2·0 ml. NHSS. After centrifugation the supernatant suspension was treated for five minutes with one-third of its volume of anaesthetic ether, recentrifuged, and the aqueous layer removed and inoculated on the chorioallantois (in the case of the tail) or intraperitoneally in mice. Sometimes urine was collected when the mice voided during the anaesthetic and it was then inoculated on the chorioallantois.

The sera were examined for ectromelia antihaemagglutinin against four agglutinating doses of virus and the results are expressed as the reciprocal of the initial dilution of serum in red blood cells (before the addition of virus) which gave "+" agglutination.

Apart from the animals killed at various stages of the experiment, nine mice died of acute ectromelia, three on each of the fifth and sixth days after inoculation, two on the eighth day and one on the ninth day.

In Table 1 the results are expressed by a figure which represents the order of the concentration of the virus; namely the logarithm of the number of virus particles per ml. in the suspension under consideration. Sometimes sufficiently low or high dilutions to obtain a satisfactory end point were not tested, and this is indicated by "<3", if for example no foci were detected in the lowest dilution used, 10^{-2}, and ">4" or ">6" when confluent lesions were present in material tested only in low dilutions (undiluted or 10^{-2}).

TABLE 1.

Concentration of virus in various organs and tissues at intervals after inoculation of "Hampstead" ectromelia in the tail, expressed at the logarithm of the tenth power of the concentration in the suspension examined.

Time after infection.	E-AHA of serum.	Blood. Plasma.	Blood. Washed RBC.	Tail.	Inguinal lymph nodes.	Kidneys.	Liver.	Spleen.	Lungs.	Nasal mucosa.	Testis.	Urine.	Subinoculation in mice.† Faeces.	Subinoculation in mice.† Small intestine.	Local lesion in tail.* Swelling.	Local lesion in tail.* Scabbing.
4 hours	..	2	0	5	0	..	..	..	..	..	..	..	..	..	—	—
24 hours	0	0	0	3	0	0	0	0	0	0	0	..	0	0	—	—
2 days	0	0	0	3	0	0	2	1	0	0	0	0	0	0	—	—
3 days	0	0	0	>4‡	0	0	2	0	0	0	0	0	0	0	—	—
4 days	0	4	4	>5	4	3	7	8	6	0	0	0	0	+	—	—
5 days	10	3	2	4	5	0	6	8	3	1	0	0	0	+	+	—
6 days	30	0	0	>6	5	2	<3‡	<3	0	3	0	..	0	+	++	+
7 days	40	0	0	>6	0	0	<3	<3	2	0	0	..	0	0	+++	+
14 days	1280	..	..	7	..	0	0	4	..	..	..	..	..	..	++	++
21 days	640	..	..	4	0	0	0	0	0	0	..	..	..	..	+	+++

* Amount of swelling or scabbing of tail varied from "—" (none) to "+++".
† Presence or absence of virus expressed by "+" or "0".
‡ Satisfactory dilutions to obtain an end point were not examined.

The Table shows that after inoculation of a large dose the virus is present in the blood and in high concentration in many organs on the fourth and fifth days, but thereafter, as the antibody titre rises the virus can be demonstrated only in a few organs and finally only in the skin of the tail, upon which scabby lesions were still present at the end of three weeks. With smaller doses the sequence of events is somewhat delayed.

DURATION OF INFECTIVITY OF INFECTED MICE.

An experiment was designed similar to that described in a previous paper (Fenner, 1947b, Table 3). Six mice were inoculated in the pad of the left hind foot with 0·05 ml. 10^{-7} "Moscow" ectromelia virus. After one day they were transferred to a clean cage and three normal mice were added to this cage. On the third day, and at intervals of two days thereafter until

the twenty-third day, the survivors of the six inoculated mice were transferred to a clean cage along with three fresh normal mice and the normal contact mice which had been exposed to infection were placed in a clean cage. Three other normal mice were added to the recently vacated soiled cage, and they were transferred to a clean cage after three days' exposure. All surviving contact mice were killed fourteen days after their first exposure. This gave an indication of the duration of infectivity of the original inoculated mice; and of the relative infectivity of the mice in their cage, and the soiled and possibly contaminated cages. The results are shown in Table 2.

TABLE 2.

Infectivity of mice inoculated intradermally with "Moscow" ectromelia virus.

Day.	Condition of inoculated mice.*	Fate of contacts added with mice.	Fate of contacts added to soiled cage.
2–3	Normal (6)	0/3†	0/3†
4–5	Normal (6)	0/3	0/3
6–7	Slightly swollen feet (2). Normal (4)	1/3	0/3
8–9	Died of acute ectromelia, feet not swollen (4). Foot considerably swoilen (1). Foot slightly swollen (1)	0/3	0/3
10–11	Foot very swollen (1). Foot slightly swollen, conjunctivitis (1)	3/3	2/3
12–13	Foot very swollen (1). Foot considerably swollen, conjunctivitis (1)	3/3	1/3
14–15	Foot very swollen and scabbed (1). Foot swollen and scabbed, conjunctivitis (1) Both showed early generalized secondary rash	3/3	3/3
16–17	Very swollen, scabbed feet, severe generalized scabbing (2) Conjunctivitis (1)	3/3	1/3
18–19	Very swollen, scabbed feet, severe generalized scabbing (2) Conjunctivitis (1)	2/3	0/3
20–21	Small, thin animals. Conjunctivitis healed. Generalized rash healing (2)	1/3	0/3
22–23	Generalized rash healed with scarring and loss of hair. Still firmly adherent scabs on inoculated feet (2)	0/3	0/3
24–41	Scabs on foot during early stages, otherwise complete healing and recovery (2)	0/3	0/3

(1) Number of inoculated mice conforming with given description shown in brackets.
(2) Numerator = number of mice infected, denominator = number exposed.

One of the inoculated mice died of acute ectromelia on the eighth day and three on the ninth day. The inoculated foot of one of these mice was slightly swollen, in the others there was no local lesion whatever. This experiment demonstrates that after pad inoculation with a small dose of virus, mice first become infective about the seventh day, although only small amounts of virus could have been liberated at this stage, for only one of three contact mice was infected, and it showed only serological evidence of infection. Although four mice died of acute ectromelia during the next two days, no contact mice were infected. However, from the tenth day onwards, when the feet of the surviving mice were swollen and scabbed, conjunctivitis occurred in one and a generalized rash appeared (on the fourteenth day), all contact mice were infected and the cage was sufficiently contaminated to allow infection of a proportion of mice added to the soiled cage. Healing of the skin rash, the conjunctivitis and the inoculated feet commenced on the nineteenth day, and although scabs were present on the feet until the end of the experiment, only one contact animal was infected on the twentieth day and none thereafter. Numerous other experiments confirm that mice cease to be infective on recovery from an attack of ectromelia.

ELIMINATION OF THE VIRUS.

Infection with ectromelia occurs through minute abrasions of the skin, and mice may be infected by exposure to a contaminated environment as well as by exposure to infected cage mates. The important feature appears to be the superficial contamination of solid material in the environment, including the bodies of the mice themselves. Under ordinary circumstances elimination of virus in the faeces, the urine, from skin lesions, in conjunctival and nasal discharges, and in the saliva may be important in effecting this contamination, and under special circumstances discharge of virus in the milk or in the semen might be of some importance in the transfer of virus from one animal to another. The main problem is to determine what form of elimination provides the largest proportion of this environmental contamination.

Preliminary experiments indicated that virus could be found at times in the faeces, the urine, washings from the nose and mouth, conjunctival discharge and material from lesions of the skin. In keeping with the evidence provided in Table 2, that infected mice are not infective in the early stages of infection, nor after recovery at the end of the third week, virus was not found in discharges collected during these periods.

Several formal experiments were carried out, testing the discharges taken from individual mice at various periods after inoculation. Washings were usually made with a Pasteur pipette from the pad of a normal hind foot to control possible surface contamination with virus. In many instances, small amounts of virus were found in such washings taken from infected mice which had been running in cages which had not been cleaned for two or three days. Faeces were collected as passed in response to gentle rubbing of the abdominal wall, repeated several times through the day. Attempts were made to collect urine in a similar way, but as the experiments were carried out in hot weather they were unsuccessful.

Nasal washings were made with a Pasteur pipette by repeatedly drawing up and expelling a small drop of saline containing penicillin and sodium sulphadiazine, placed on the nares, until it became cloudy; and mouth washings and washings from the surface of skin lesions and the eyes were collected in a similar way. Each drop of washings was mixed with 0·5 ml. of saline containing penicillin and sodium sulphadiazine and inoculated in eggs or mice. Occasionally the animal was killed after the washings had been taken and sections taken from various organs. In one experiment, samples of blood were collected from the tail veins in a similar way, using a pipette previously moistened with citrate solution and discharging the blood collected in 0·5 ml. sodium citrate solution. The results of experiments on excretion of virus are set out in Tables 3, 4 and 5.

TABLE 3.

Isolation of ectromelia virus from various discharges of infected mice.

Period of disease.	Faeces.	Urine.	Nasal washings (including mouth washings).	Conjunctival washings.	Washings from scabs.
Acute fatal cases (death before tenth day)	2/2*	13/38 (a)	1/3 (b)	2/2 (c)	1/1 (d)
Active cases killed between tenth and twenty-first days	0/2	3/16 (e)	10/17 (f)	5/7 (g)	12/12
Recovered mice (after twenty-first day)	0/2	0/13	0/8	—	1/6

* Numerator indicates number of positive results, denominator indicates number of animals tested.

(a) Five of positive results obtained from animals dying after intraperitoneal inoculation, with enlarged kidneys but no focal haemorrhages. In seven of the other eight positive cases the kidneys showed focal haemorrhages, after inoculation by various routes.

(b) Positive results obtained from an animal inoculated intranasally without an anaesthetic.

(c) Both mice had conjunctivitis.

(d) Primary lesion of belly wall.

(e) Positive results obtained only from animals with haemorrhagic lesions of the kidneys.

(f) Five of the ten mice in which virus was obtained in the nasal washings had skin lesions of the external nares.

(g) All mice examined had conjunctivitis.

TABLE 4.

Elimination of ectromelia virus from mice infected with "Hampstead" ectromelia 10–2 by pad inoculation (pooled material from four mice).

Day of disease.	Condition of mice.	Blood.	Faeces.	Nasal washings.	Mouth washings.	Washings from surface of infected foot.	Washings from surface of normal foot.
6	feet swollen, not scabbed	+	+	+	+	..	..
8	feet swollen, not scabbed	+	+	+	+	+	..
10	feet swollen, scabbed	—	—	+	+	+	..
12	feet swollen, scabbed	+	—	—	—	+	—
14	feet swollen, scabbed	..	—	—	—	+	—

TABLE 5.

Elimination of ectromelia virus at various intervals after pad inoculation.

Strain of virus.	Day after inoculation.	Condition of mice.	Faeces.		Nasal washings.		Mouth washings.		Washings from skin lesions.		Washings from normal foot.	
			Result.*	Concentration.†	Result.	Concentration.	Result.	Concentration.	Result.	Concentration.	Result.	Concentration.
"Hampstead" 10^{-5}	12	early generalized rash	2/4	10^3	3/4	10^3	4/4	10^3			4/4	10^3
"Moscow" 10^{-6}	12	early generalized rash	1/3	10^3	3/3	10^3	3/3	10^3			3/3	10^3
"Moscow"	15	extensive general. rash	0/3		1/3‡	10^3	1/3	10^3	3/3	10^6	1/3	10^3
"Moscow"	8	well-developed primary lesion	0/1		1/1‡	10^3	1/1	10^3	1/1	10^8	0/1	
"Moscow"	11	inoculated feet very swollen and scabbed	1/6	10^3			6/6¶	10^4	6/6	10^7	5/6	10^3
Totals			4/17		8/11		15/17		10/10		13/17	

* Numerator = number of positive results; denominator = number of animals in group.

† Concentration expressed as approximate number of infective particles per ml. of fluid used for washings.

‡ Scrapings of nasal mucosa.

¶ Nasal and mouth washings pooled.

Virus was isolated with the greatest regularity and in the greatest amount in washings from skin lesions, whether of the pad of the foot, in the hairy parts of the body, or of the eyelids. Smaller amounts of virus were obtained at times in the nasal and mouth washings, in the urine, and in the faeces. The evidence derived from histological study of various organs and tests for virus in the excretions can be correlated to give a fairly complete picture of the methods of elimination of ectromelia virus.

Skin and Conjunctival Lesions.

In an earlier paper (Fenner, 1947b) the primary skin lesion and the generalized secondary rash of ectromelia were described. When sections are made of any of these skin lesions in the early stages, before extensive necrosis and scabbing obscures the picture, numerous inclusion bodies can be seen in the epithelial cells. Sections of the eyelids of mice with conjunctivitis showed a similar picture. Washings from such lesions, taken before the twenty-first day, always contained large amounts of virus.

In the natural disease, primary and secondary skin lesions and conjunctivitis are all common lesions. For instance, in one experiment with "Moscow" ectromelia spreading in a herd of 97 mice, primary lesions were found within three weeks of exposure to infection in 72, an extensive secondary rash occurred in 42 out of 46 survivors and conjunctivitis in 23 of the 51 fatal cases and 12 of the survivors. There is little doubt that the skin lesions and conjunctivitis described by Kikuth and Gönnert (1940) in a colony of mice (strain Dö, p. 306) with "latent" ectromelia were similar rashes and conjunctival lesions due to ectromelia.

Thus the concentration of virus in discharges from both primary and secondary skin lesions is sufficiently high, and the lesions are sufficiently common, to account for the major part of contamination of the environment and passage of infection from diseased to healthy mice.

Nasal Mucosa.

The interpretation of isolation of virus in washings from the nares is complicated by a number of factors. Firstly, as Table 5 indicates, small amounts of virus can often be isolated from the surface of normal feet of infected mice. Mice use their noses as actively in the investigation of their environment as they use their feet for running about their cages, and surface contamination of the nares is equally likely. Secondly, both primary lesions and lesions of the secondary rash sometimes occur on the nares, and such lesions were noted in five out of ten cases in which virus was isolated from nasal washings (Table 3). Thirdly, nasal washings, whether collected by drawing fluid placed on the external nares up and down in a Pasteur pipette, or by immersing of the nares of an anaesthetized mouse in a pool of saline, are often contaminated by material from the oral cavity, and this may contain virus.

All these facts minimize the importance of the positive results obtained, and histological examination of the nasal mucosa provides little evidence of multiplication of virus in this area. The only sections in which necrosis and inclusion bodies were seen in the nasal mucosa were those made from animals inoculated intranasally without anaesthetization, and the nasal exudate from these animals always contained virus. However, such infections which cause a nasal discharge and sneezing never occur in the naturally-spreading disease. Evidently the nasal mucosa is unimportant either as a portal of entry of the virus or as a route of elimination.

Oral Cavity.

Washings from the oral cavity taken before nasal washings (to avoid contamination from the nasal cavities) often contained virus (Table 5). Surface contamination was not a factor in these cases and it seems certain that virus is actually present in the saliva. Two sources are suggested. Firstly, there may be an enanthem of the tongue and buccal mucosa occurring simultaneously with the exanthem. I have not detected this by direct examination with a binocular dissecting microscope, but such lesions may sometimes occur.[2] Secondly, the virus may be excreted in the saliva. Several observers, e.g. Marchal (1930), have described the presence of inclusion bodies in the cells of the salivary glands. While secretory granules may cause confusion (e.g. Duff and Corbett, 1945) true inclusion bodies do sometimes occur, and we have isolated virus from such glands by subinoculation.

The excretion of virus in saliva may have some importance in transmission of infection when many mice use a few overhead drinking vessels, for the primary lesions often occur on the snout or lower lip.

Faeces.

Localized lesions of the intestinal tract with inclusion bodies and cellular necrosis were described by Marchal (1930) and Greenwood *et al.* (1936). The latter authors prepared sections from the small intestines of 162 animals which died of acute ectromelia with characteristic lesions of the liver and spleen and found inclusion bodies near the lymphoid follicles in 63 p.c. In the few sections examined, our experience has been similar. The nature of the lesions suggests that virus might be liberated into the lumen of the gut by the necrosis of epithelial cells and passed in the faeces.

Inclusion bodies also occur occasionally in cells of the pancreas, and virus liberated from them may pass with pancreatic secretions into the intestine.

Tables 3, 4 and 5 indicate that occasionally small amounts of virus are passed in the faeces. Its source may be either the pancreas or the lesions of the intestine.

Occasionally, especially in young mice with severe generalized skin and visceral lesions, diarrhoea occurs. The highest concentration of virus isolated from the faeces was from the diarrhoeal contents of the rectum of such a mouse.

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2 Small ulcers with characteristic inclusion bodies in the neighbouring epithelial cells have since been found frequently on the tongue and rarely on the buccal unicord in animals with severe secondary rashes.

Urine.

Marchal (1930) described involvement of the kidneys as a comparatively rare feature of ectromelia. After intraperitoneal inoculation, the kidneys are often swollen, without specific focal invasion, and the urine from such animals often contains virus (Table 3). In the natural disease or after intradermal inoculation, such enlargement of the kidneys without focal necrosis rarely occurs, but occasionally both kidneys are enlarged and covered with small haemorrhagic spots. With "Moscow" ectromelia this occurred in 7 out of 84 animals after intraperitoneal inoculation, in 5 out of 80 after pad inoculation, and in 7 out of 51 animals dying in the course of an epidemic amongst 97 mice. Sections of such kidneys show widespread focal necrosis, especially in the region of the convoluted tubules. Mann's stain shows intracellular inclusion bodies in the epithelial cells near the necrotic foci, and cellular debris and eosinophilic globular bodies can be seen in the lumina of the tubules. In the bladder of one animal whose kidneys were severely involved there was a small focus of superficial necrosis of the mucosa with ectromelia inclusion bodies within the epithelial cells, but this is the only case of infection of the bladder yet found. Most of the positive results obtained from the urine of animals in the active stage of the disease (Table 3) were from the bladders of mice with such lesions of the kidneys but occasionally virus was recovered from the urine of mice with macroscopically normal kidneys. The highest concentration of virus in the urine was about 5,000 infective particles per ml.

Semen.

General observations suggest that sexual intercourse would be of very slight importance in the transmission of ectromelia, since virus is freely liberated from skin lesions and infection occurs readily through small lesions of the skin. It was shown earlier (Fenner, 1947b) that infection could occur through the vaginal mucous membrane, and very small amounts of virus were found in the semen of three out of four acute fatal cases. The theoretical possibility of sexual transmission from the male to the female thus exists, but must be of minor importance. I have not investigated the possibility of the reverse process, infection of the male by the female.

Milk

As was pointed out in a previous paper (Fenner, 1947b) virus can be isolated from milk obtained from the stomachs of young mice which are nursed by infected lactating females. It is difficult to assess the importance of this mode of excretion of virus in the infection of nursling mice, for such young mice rarely develop local skin lesions after scarification with virus, and it is therefore impossible to decide whether infection has occurred in the way usual amongst adult animals, i.e., through abrasions of the skin. In an experiment in which nineteen pregnant mice were inoculated intradermally with small doses of virus, 50 deaths of nursling mice, due to ectromelia, occurred in fifteen families, and in only one nursling animal was a primary skin lesion found. When a nursing mother developed a secondary rash it usually involved the nipples, which became hard and ceased to be effective for suckling. Section of such a nipple and underlying mammary gland showed characteristic widespread necrosis of the surface epithelium, but no involvement of the mammary gland. The lesions examined were late ones, and only an occasional inclusion body could be seen in the epithelial cells at the periphery. The young of such mice died of starvation, as they were unable to obtain any milk.

In the special case, which must be very rare in natural enzootic ectromelia, of infection of a mother just before or just after birth of its young, excretion of virus in the milk may be a factor in the infection of nursling mice.

SURVIVAL OF VIRUS AFTER EXCRETION.

The experiment described in Table 2 indicates that cages recently vacated by infective mice are still infective. A few experiments were carried out on the heat stability of the virus and on the duration of infectivity of cages containing litter which had been artificially contaminated with virus.

Marchal (1930) reported that virus survived for 10 minutes after heating at 55° C., but not for 30 minutes. Commencing with egg membrane virus of a titre of 10^7 infective particles per ml., we found living virus present 60 but not 70 minutes after heating at 55° C.

Two ml. of this preparation of virus were diluted 1:2 and sprayed over the sawdust litter of the floor of a number of cages. At intervals of three, sixteen, twenty-four and forty-eight hours after contamination of the cages, four mice, two of which had been scratched on the belly and hind feet with a steel needle, were placed in the cages. Fourteen days later all these mice were killed. Three of the four mice placed in a cage three hours after the virus died of acute ectromelia, and the fourth was not infected. In each of the cages treated with virus sixteen and

twenty-four hours earlier one scarified mouse was infected, and none of the mice in the cage treated forty-eight hours before was infected.

To see how long virus which had been dried at room temperature remained viable, 0·2 ml. of the undiluted suspension was spread over the bottom of each of a number of Petri dishes and dried and stored at room temperature, which varied between 15° C. and 20° C. At various intervals afterwards, the dried material was resuspended in 1 ml. saline and 0·4 ml. of this suspension inoculated intraperitoneally in mice. Viable virus was present 8 but not 9 weeks after drying, and regular chorioallantoic titration of the reconstituted material at weekly intervals showed a logarithmic decline in the number of viable particles.

These experiments, and the observations of the relative frequency of infection in contaminated cages in the presence or absence of the infective mice, show that although the virus is relatively resistant to heat and drying, and infection can be spread by "fomites," the infected mouse with a primary lesion or secondary rash is much more efficient in disseminating the disease to other mice by actual contact.

DISCUSSION.

The series of investigations of which this forms the third was commenced to clarify certain aspects of the epidemiology of ectromelia before using the infection for experimental epidemiology. Several features of the disease which are important in the interpretation of experimental epidemics have been studied. Infection occurs through small abrasions of the skin, often with the production of a local skin lesion. A large amount of virus is liberated in the discharge from the primary lesion when this ulcerates. Blood stream dissemination always occurs, often with the production of severe necrotic lesions in the liver and spleen and lesions in many other organs. In animals which survive the acute "abdominal" disease, as McGaughey and Whitehead (1933) call it, an extensive rash occurs over the whole body. This exanthem rapidly progresses to ulceration and scabbing, and large amounts of virus are eliminated in the discharges from these skin lesions, which constitute the main method of passage of infection to uninfected animals. The virus that is liberated is rather resistant to heat and to drying, and retains its infectivity for some days after being shed. Thus infection can occur from contaminated litter or via the contaminated hands of a laboratory attendant. Other methods of elimination of virus exist but are less important in both the frequency of their occurrence and the concentration of virus eliminated than are the skin lesions.

In consequence of this infected animals eliminate virus only during the short period of illness between (with small infective doses) about the seventh and twenty-first day. Animals dying of acute ectromelia are of less importance in the infection of their cage mates, unless they are eaten by them, than are the survivors which develop a secondary rash. Even with the few animals in which this rash has not healed by the twenty-first day, infectivity has ceased, probably because the virus present in the lesions is inactivated by antibody.

It is hard to assess Greenwood's (1936) figures on duration of infectivity of immunized and infected mice, for he worked in ignorance of the mode of transmission of the disease and death from ectromelia was his only available index of infection—an inadequate criterion for such an experiment.

Burnet's (1945 and 1946) discovery that ectromelia was related to vaccinia has been confirmed, and a consideration of the natural history of the disease leaves no doubt that it is the murine representative of the mammalian pox diseases. Marchal originally proposed the name "infectious ectromelia" on account of the frequency with which amputation of the extremities occurred. Few subsequent observers have found this at all common except after pad inoculation of young mice. In view of this and of the newly found close relationship of the disease to the mammalian pox diseases, Professor F. M. Burnet has suggested that "mousepox" should be used as a synonym for "infectious ectromelia."

SUMMARY.

Mice infected with ectromelia are infective only during a short period preceding death in acute fatal cases and between the first appearance of the primary skin lesion, on about the seventh day after infection, and healing of the secondary rash on about the twenty-first day in cases which survive.

During this infective period, virus may be eliminated in the faeces, urine, saliva, conjunctival discharge and the discharge from skin lesions. From the points of view of frequency of occurrence and amount of virus eliminated, skin and eye lesions far exceed in importance the other avenues of elimination.

The virus is relatively heat resistant and when shed into the environment that contaminated environment remains infective to mice which come in contact with it for at least twenty-four hours. Virus can frequently be isolated from the surface of the normal skin of mice living in the contaminated environment.

It is suggested that as ectromelia is the true murine representative of the mammalian pox diseases, the name "mouse-pox" should be used as a synonym for ectromelia.

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MUMPS

III. IMMUNIZATION EXPERIMENTS IN MAN

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Investigations with mumps virus were started in this laboratory following reports on cultivation of the virus in the chick embryo by Habel (1945). Shortly afterwards Levens and Enders ('945) reported observations on haemagglutination by amniotic fluid containing mumps virus. Our work on isolation and cultivation of four strains of the virus in the chick embryo and on haemagglutination and serology have already been reported (Beveridge, Lind and Anderson, 1946; Beveridge and Lind, 1946). Further work has been concerned mainly on the one hand with cultivation of the virus in the allantoic cavity with the object of developing a practicable method of producing vaccine, and on the other hand with vaccination experiments in human subjects.

CULTIVATION OF THE VIRUS IN THE ALLANTOIC CAVITY.

Effect of Seed Virus Used.

We have not attempted to cultivate any strains in the allantoic cavity until they have had at least several passages in the amniotic cavity. There was a large variation in the growth of the virus from egg to egg, therefore, we used six to ten eggs for each of the early passages and used for transfer the fluid with the highest titre. Improved growth of the virus was usually obtained with each of the first allantoic passages; probably at least eight to ten passages are required for the virus to become fully adapted.

The ease with which different strains become adapted to growth in the allantoic cavity varies, and so does the amount of virus produced after they have been through ten passages and apparently have reached their maximum adaptation. In our experience most strains continued to give inconsistent and usually poor yields of virus. In this respect our strain MEL has been much superior to our other strains but the most consistent results have been obtained with a strain kindly forwarded to us by Dr. J. F. Enders. We have designated this strain END.

With influenza virus better yields are obtained after inoculation with allantoic fluid virus diluted 1/100 to 1/10,000 rather than with undiluted fluid (Henle and Henle, 1944). We have attempted several times to demonstrate this phenomenon with mumps virus, sometimes using seed which had been kept for two weeks in the refrigerator. Only once did the results favour the seed diluted 1/100; on all other occasions there was no difference, or undiluted seed gave somewhat better yields. Seed diluted 1/1,000 usually gave poor results.

Comparative tests using as seed freshly harvested virus and virus kept ten days in the refrigerator showed no differences in yield. A comparative test using as seed (a) amniotic fluid virus harvested after two days' incubation, or (b) allantoic fluid virus with the same titre harvested after five days' incubation, showed no difference in the yield obtained. Usually amniotic fluid virus was harvested after two or three days' incubation and allantoic fluid intended for seed after three or four days, but we have seen no indication that the period of incubation for growth of seed virus, whether it is allantoic or amniotic fluid, has any effect on the yield of virus subsequently obtained. The best yields were obtained when high titre seed was used.

Age of Embryos When Inoculated.

Comparative tests showed that the highest titres were obtained by inoculation of seven-day embryos, and that as the age of the embryos at the time of inoculation increased, there was a considerable decrease in the titres obtained. However, the volume of allantoic fluid harvested

[1] This work was carried out under a grant from the National Health and Medical Research Council.

from embryos inoculated at seven days was considerably less than that from eggs inoculated at eight or nine days. Usually embryos inoculated at eight days and incubated a further four or five days at 35° C. yielded from 4–10 ml. of fluid and this was regarded as satisfactory.

Temperature and Period of Incubation.

Comparative tests showed that, when the eggs were incubated at 33° C. after inoculation, the titres were about twice as high as when incubation was at 35° C. but only about half the volume of allantoic fluid was obtained. At 36° C. the titres were lower still and the volumes greater than at 35° C. When the other conditions were propitious, satisfactory volumes and titres were obtained at 35° C. and this temperature was adopted as a routine.

Before inoculation the eggs were incubated at 99·5° F. (37·5° C.). If the eggs were incubated at a temperature higher than 100° F. (37·8° C.) before inoculation poorer yields of allantoic fluid were obtained.

When undiluted allantoic fluid virus from a strain which had had at least ten passages in the allantoic cavity was inoculated into nine-day embryos there was no increase in titre between the third and fourth day of incubation at 35° C. However, when the embryos were inoculated on the eighth day and then incubated at 35° C. there was some increase in titre between the third and fourth day, and when seeded with allantoic fluid virus diluted 1/100 the titre sometimes increased up to the fifth day.

Other Factors.

Unexplained differences occurred in the results from time to time and it was thought that changes which had been made in the diet of the fowls producing the eggs may have been the cause. Two dietary factors have been investigated for such an effect. Eggs from hens fed an ample amount of green feed were compared with eggs from hens which had received no green feed for two weeks. In the second test, eggs from hens fed on a diet containing a considerable amount of finely chopped lucerne were compared with eggs from fowls receiving no lucerne. In neither trial was there any difference in the growth of the virus in eggs of the different groups.

With strain END, and to a slightly less extent with MEL, good yields of virus were fairly consistently obtained if the various conditions listed above were optimal. With our other strains the results varied greatly from day to day for no reason which we have been able to discover. Many minor points of technique in inoculation and handling of the eggs and the ventilation of the incubated eggs before and after inoculation have been investigated without discovering the cause of these irregularities. However, there was some indication that poorer results may be obtained with eggs which before inoculation were in a crowded incubator with ventilation at a minimum. Inoculation of para-aminobenzoic acid or ascorbic acid had no significant effect on the yield of virus although eggs inoculated with ascorbic acid gave slightly poorer yields.

PREPARATION OF VACCINE.

Technique of Preparation.

Embryos after eight days' incubation at 99·5° F. are prepared for inoculation by drilling a groove in the shell immediately over the embryo. They are then inoculated into the allantoic cavity with a pipette and needle as described by Burnet and Beveridge (1943). With strain END the seed is used diluted 1/100 in broth but with our strains the allantoic fluid virus is used undiluted or diluted only 1/10. The eggs are incubated at 34·5–35° C. for four or five days and then chilled in the refrigerator for at least two hours before harvesting. With each batch of eggs we usually harvest the first dozen into separate test tubes and "spot test" each by placing one drop of fluid in a tube and testing for haemagglutination. If all fluids give complete agglutination (one drop of fluid to 0·25 ml. 2 p.c. fowl cells) and they have the "smoky" appearance characteristic of good titre fluids, the rest of the batch are harvested directly into 25 ml. screw cap bottles. If, on the other hand, all of the first dozen eggs do not yield satisfactory fluids, it may be necessary to harvest all eggs separately and pool only those which by "spot test" and smoky appearance are judged as suitable.

After preliminary tests for sterility on blood agar, the fluids are formalinized at 0·1 p.c. and screw caps put on the bottles. The bottles are immersed up to the neck in a water bath at 37° C. for six hours, samples are taken and the bottles are left overnight in the refrigerator and the next day placed at 37° C. for a further six hours. The samples taken after the first six hours at 37° C. are tested for sterility, titrated by haemagglutination and tested for absence of active virus by amniotic inoculation of six 10-day embryos. For inactivity tests usually the samples from several bottles are pooled. The vaccine is used if the initial haemagglutination titre is at least 30 and if at least three of the six inoculated eggs survive six days and show no haemagglutinin in the amniotic fluid.

Before the vaccine is issued 0·25 p.c. phenol is added. This prevents the pain which the formalin otherwise causes on injection and at the same time serves as a bacteriostatic agent. After the addition of phenol the haemagglutinin titre of the vaccine falls much more rapidly than in the case of unphenolized material although the antigenicity of the vaccine is not affected. If the vaccine is to be stored for a period the phenol is not added until the vaccine is about to be issued.

The Effect of Storage on Mumps Vaccine.

The haemagglutination titre of formalinized allantoic (and amniotic) fluid from strain WAL increased on storage at 4° C. This increase varied from batch to batch but usually a two-fold rise occurred during the first two weeks and often a further two-fold rise during the next two weeks. This phenomenon indicates, as also does the slight opacity or "smokiness" of the fluids, that the initial haemagglutination titre of mumps fluid represents a much greater virus content than do corresponding haemagglutination titres of influenza virus.

Possible explanations of this phenomenon are: (1) disaggregation of clumps of virus on storage, (2) destruction of some substance masking the receptors of many of the virus particles, (3) destruction of an inhibitory substance present. Attempts have been made to bring about this increase in titre by means other than storage. These include treatment with urea, alterations of pH, dialysis and prolonged shaking, but all were unsuccessful.

Unlike vaccines prepared with WAL strain those prepared with END strain showed no increase in haemagglutinin titre after storage at 4° C. The majority of END vaccines showed a progressive decrease in haemagglutinin titre after several months, although in a few cases only a slight fall occurred and in others no fall in titre was observed over a period of eight months.

It was decided to test the efficacy of vaccines stored for various periods of time and to include in the test some which showed little or no diminution in titre and others from which the haemagglutinin had almost disappeared. Rabbits were accordingly inoculated intravenously with 2 ml. of such vaccines. They were bled at the time of inoculation (Serum I) and fourteen days later (Serum II). The sera were titrated in parallel by Burnet's (1946) method. The results are shown in Table 1 and indicate that, except in one case, vaccine stored up to five months is still antigenic, independent of its haemagglutinin titre.

TABLE 1.

The effect of storage on the antigenicity of mumps vaccine.

Vaccine batch No.	Original titre.	Age of vaccine.	Titre at time of inoculation.	Rabbit No.	Antibody titres. SI.	SII.
176	160	1 week	160	1	<10	240
117	20	3 months	20	2	<10	240
60	80	5 months	80	3	<10	320
68	40	5 months	<10	4	40	600
83	40	5 months	<10	5	50	600
93	50	5 months	<10	6	80	100

SEROLOGICAL STUDIES.

Technique.

The method of titration of antibody previously described (Beveridge and Lind, 1946) using 2 p.c. guinea-pig cells is satisfactory for measuring increases after an attack of the disease. However, by this method nearly all sera from persons who give no history of having had mumps show some degree of nonspecific inhibition and it is usually not possible to determine from a specimen of serum whether a person has had mumps or not. Attempts have been made to devise a more sensitive test. Somewhat wider differences were obtained between nonspecific titres and specific antibody titres with a test in which (a) the virus and serum dilutions were allowed to react for one hour at 35° C. before adding the red cells, (b) only one haemagglutinating dose of virus (estimated by the usual method with 2 p.c. cells) was employed, and (c) 0·5 p.c. cells either guinea-pig or fowl were used and allowed to settle in the refrigerator. However, a more sensitive method of detecting low levels of specific antibody has become available in the method described by Burnet (1946) involving agglutination of human group O red cells which have been "sensitized" by contact with mumps virus. After elution of virus from the cells they are agglutinated to high titre by specific antibody. This technique is now used for studying the response to vaccination. From a single specimen of serum it is often possible to determine whether or not a person has had mumps according to whether the titre is very high

or very low, but some specimens give a moderate titre and it is not possible at present to interpret these.

As has been reported, the first three strains which we isolated appeared to be identical serologically. The other two strains MEL and SOM have not been examined serologically by preparation of antisera in rabbits, but with human sera they give results similar to those obtained with the other strains.

When strain END arrived from U.S.A. we inoculated it into rabbits intravenously in the same way as we had with our first three strains, that is, one injection of 1 ml. of active virus. Table 2 shows the results of haemagglutinin-inhibition tests with guinea-pig cells comparing END and WAL antisera and END, WAL and STO viruses. These strains appear to be serologically similar although the results suggest that END is not identical with STO and WAL.

TABLE 2.

Cross serology of mumps strains.

	Virus.		
	END.	WAL.	STO.
END antiserum	30–480	20–160	30–140
WAL antiserum	40–640	90–960	30–750
STO antiserum	40–480	30–640	40–480

IMMUNIZATION EXPERIMENTS.

Experiment 1.

Eight children from eight to thirteen years of age were selected as not having had mumps according to the history given by their parents. They were public hospital patients mostly convalescing from poliomyelitis. They were inoculated subcutaneously with 2 ml. vaccine prepared from allantoic fluid as described above. Three weeks later a second similar injection was given. Samples of blood were taken at the time of each injection and two weeks after the last one. These sera were titrated by inhibition of agglutination using guinea-pig cells as described by Beveridge and Lind (1946) and by the method of Burnet (1946) involving agglutination of human O cells sensitized by contact with mumps virus. The results are shown in Table 3. It will be seen that only one child, BUR, showed any appreciable effect from the second inoculation.

TABLE 3.

		Inhibition of agglutination.			Agglutination of sensitized cells.		
	Age. yrs.	SI.	SII.	SIII.	SI.	SII.	SIII.
BAR	8	15	36	33	240	2,400	1,200
MUN	13	10	46	40	160	1,600	1,600
NOR	11	20	105	110	320	>3,200	3,200
WIL	12	20	55	40	160	2,400	2,400
KEL	11	15	28	30	30	600	600
BUR	13	15	20	40	<10	150	400
BAT	10	13	24	33	<10	800	800
PRA	11	13	105		30	3,200	
SOM*					<10	3,200	

* Acute and convalescent sera from a case of mumps are shown for comparison.

Experiment 2.

This group consisted of 24 boarders from a boys' public school. Their ages ranged from 10 to 17 years. According to the history given by their parents none had previously had mumps. Each boy was inoculated subcutaneously with 2 ml. of allantoic fluid vaccine and this was followed three weeks later by a second similar injection. Samples of blood were taken at the time of each injection and two weeks after the last one. The sera were titrated simultaneously by Burnet's method. There was insufficient serum for a further titration by haemagglutination inhibition. The results are shown in Table 4. In this experiment also it is evident that a second dose of vaccine does not cause a further increase in the antibody titre.

TABLE 4.

	Age yrs.	Agglutination of "sensitized" cells. SI.	SII.	SIII.		Age yrs.	Agglutination of "sensitized" cells. SI.	SII.	SIII.
TUR	15	<10	<50	50	LOW	14	<20	<50	<50
EDM	15	<10	50	..	ACK	16	10	200	300
COL	10	<10	..	75	WRI	15	20	200	200
HUR	16	<10	<100	<50	BAR	16	20	200	200
SCA	16	<10	75	75	MAN	16	20	400	...
DUG	15	<10	50	100	COR	16	40	50	50
MIR	13	<10	50	200	LIN	17	40	200	300
HOR	14	<10	100	200	ARM	15	40	400	200
VAU	14	<10	100	200	AUC	14	40	400	400
HAR	11	<10	100	100	RIN	16	40	800	800
SCH	14	<10	200	300	BOL	12	80	600	800
MAC	15	<20	<50	<50	HAY	16	80	1,600	...
STO*				2,400					
BUR†				<10					

* Convalescent serum from a case of mumps.
† Negative control serum.

DISCUSSION.

Despite a great deal of effort it has not been possible to ascertain the conditions under which high-titre allantoic fluid virus can be consistently produced. Under laboratory conditions vaccine of adequate titre can be prepared, but our experience would suggest that large scale production would meet great difficulties.

The keeping quality of formalinized vaccine also varies considerably from batch to batch and with different strains. Antigenicity, as judged by the relatively crude test of production of antihaemagglutinin in rabbits, however, seems to be little affected by loss of haemagglutinating power. The impression we have received is that the major unfulfilled requirement is the development of a strain of mumps virus which will consistently produce high-titre fluids.

No mumps epidemics have appeared in the immunized and control groups which we have had under study and the only information that can be offered in regard to the effectiveness of formalinized vaccine is in regard to the antibody changes induced. The interpretation of antibody levels in individuals who have not suffered an overt attack of mumps or been immunized will be discussed in another paper. Here, however, it may be legitimate to draw attention to the coated cell agglutinin titres of the "first bleeding" sera in Table 3. Four of these children had relatively high initial titres and showed a very active antibody response. These must be regarded as having suffered from a subclinical infection with the virus in the past. In neither of the groups studied was there any clear response to the second inoculation, the results being similar to those described by Beveridge, Stone and Lind (1944) from studies of influenza vaccination. If clinical protection is eventually shown to be correlated with circulating antibody level, there appears to be no benefit to be gained from giving two closely spaced injections.

SUMMARY.

Although it was not possible to obtain consistent yields of high-titre mumps allantoic fluid, sufficient high-titre fluid for production of mumps vaccine on a laboratory scale was obtained by allantoic inoculation of high-titre seed virus into eight-day chick embryos and harvesting the allantoic fluid after four days' incubation at 35° C.

Mumps vaccine was prepared by an addition of 0·1 p.c. formalin to the infected allantoic fluid which was then held at 37° C. for twelve hours. 0·25 p.c. phenol was added just prior to use.

The keeping quality of different batches of vaccine varied considerably, but antigenicity seemed to be little affected by loss of haemagglutinating activity.

All strains of mumps virus used in vaccine preparation were serologically similar, although one strain END did not appear to be identical with the others.

Two doses of 2 ml. vaccine each were given subcutaneously, with an interval of three weeks between doses, to two groups of children. In general it was found that the higher the pre-vaccination antibody the greater the response to the first injection. In neither group was there any clear response to the second inoculation.

Acknowledgment. We are indebted to Professor F. M. Burnet for helpful advice and criticism throughout this work.

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THE EFFECT OF HEXOESTROL ON THE FOOD INTAKE OF SHEEP

by C. R. AUSTIN, W. K. WHITTEN, M. C. FRANKLIN AND R. L. REID

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Investigations on pregnancy toxaemia of sheep, carried out in this laboratory, have confirmed the findings of several investigators that a short period of fasting may sometimes induce the disease. It is well recognized that anorexia is a constant premonitory sign of pregnancy toxaemia which characteristically occurs during the last month of pregnancy. Whitten (1943) demonstrated that oestrogens first appear in the urine of ewes in measurable amounts at that period of pregnancy. Preliminary observations indicated that hexoestrol induced inappetance in sheep. Thus it was considered possible that oestrogens may also be the cause of an anorexia which may in turn produce pregnancy toxaemia in a manner similar to experimental starvation.

The experiments described in this paper were designed to investigate further the effects of hexoestrol on the food intake of pregnant and non-pregnant ewes. In addition, many observations were made on blood and urine chemistry, but since no changes were detected other than those associated with inanition no further reference will be made to them here.

TABLE 1.

Scheme of experiments with hexoestrol on ewes.

Group No.	Sheep No.	Initial body wt. lb.	Breed.	Treatment. Dose rate of hexoestrol.
1. Non-pregnant ewes	Y.100	85	Merino	50 mg. thrice weekly
	W.877	87	Merino	
	X.333	115	B.L.X. Romney	100 mg. thrice weekly
	M.910	88	Merino	
	175	97	B.L.X. Merino	200 mg. once weekly
	178	102	B.L.X. Merino	
	Y. 38	80	Merino	
	M.887	77	Merino	
	W.190	138	Corriedale	200 mg. thrice weekly
	W.193	125	Corriedale	
	X.223	137	Romney	40 mg. daily, increased later to 80 mg. daily
	X.224	188	Romney	
	X.228	163	Romney	
2. Pregnant ewes	M.884	80	Merino	20 mg. on alternate days
	Y.380	85	Merino	
	X.314	73	Merino	
	M.907	89	Merino	40 mg. on alternate days
	M.891	78	Merino	
	X.300	83	Merino	
3. Pregnant ewes	B. 5	77	Merino	Progressively increasing doses of 5–20 mg. daily
	B.47	79	Merino	
	B.65	88	Merino	
	B. 3	85	Merino	
	B.33	76	Merino	

Note: B.L. = Border Leicester.

EXPERIMENTAL PROCEDURE AND OBSERVATIONS.

Table 1 describes the animals used and outlines the treatment to which they were subjected.

The ration fed to all sheep consisted of wheaten chaff 44, oats 25, linseed meal 5, salt 1 and limestone 1, parts by weight. Each day, weighed quantities in excess of requirements were offered the animals, and what remained uneaten by the following morning was removed and weighed.

The hexoestrol was used in the form of a 1 p.c. solution of the free phenol in arachis oil, and was administered by subcutaneous injection.

Group 1.

Thirteen non-pregnant ewes were placed in single pens and after three weeks, when the daily food intake had become reasonably constant, the hexoestrol treatment was commenced. The dose rates and daily food consumption are shown in Fig. 1, 2 and 3. The duration of treatment was dependent to some extent upon the response of the animal.

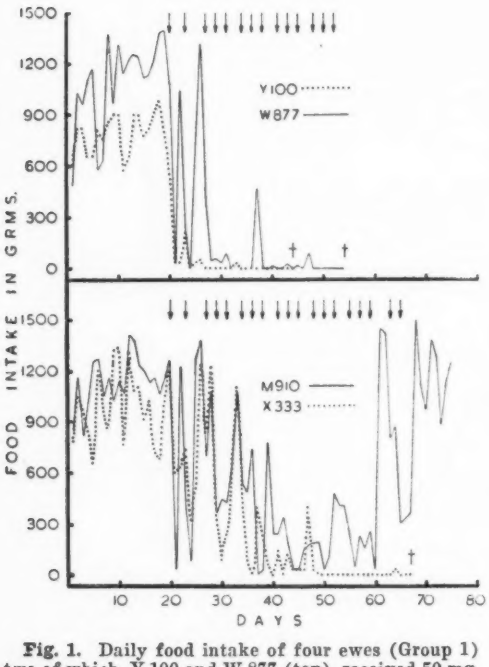

Fig. 1. Daily food intake of four ewes (Group 1) two of which, Y.100 and W.877 (top), received 50 mg. hexoestrol per dose, and the other two, M.910 and X.33 (bottom), 100 mg. per dose. The frequency of administration is indicated by the arrows.

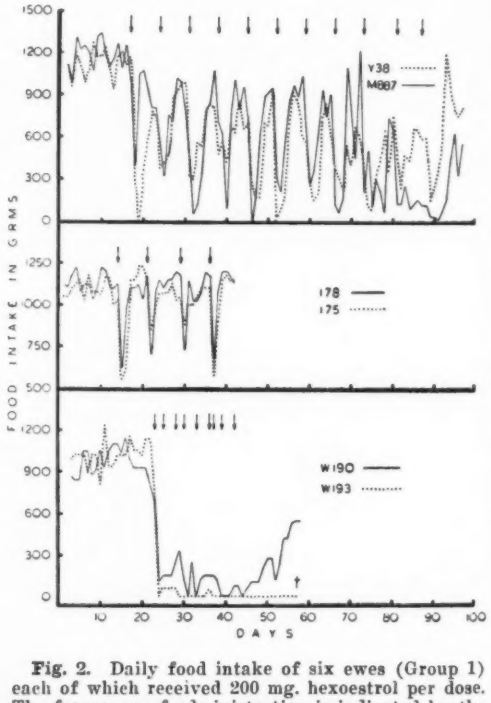

Fig. 2. Daily food intake of six ewes (Group 1) each of which received 200 mg. hexoestrol per dose. The frequency of administration is indicated by the arrows.

On the 8th day, ewe X.223 showed typical male behaviour when released for a short time with the other ewes in a sun-yard. This behaviour consisted of curling the upper lip, raising the head, smelling at urine and mounting other ewes. This phenomenon was also observed by McKenzie and Terrill (1937). The animal died 14 days after the commencement of the experiment.

Most of the sheep showed signs of mammary development and milk secretion commenced from 5–25 days after the first dose of the oestrogen.

The results, as set out in Figs. 1, 2 and 3, show clearly the immediate and often profound effect of hexoestrol on the food intake of the ewes, but considerable differences were apparent in the responses of different sheep.

Group 2.

Six ewes in the last month of pregnancy were placed in single pens. Three of these ewes, M.884, Y.380 and X.314, received 20 mg., and the other three M.907, M.897 and X.300, 40 mg. of hexoestrol in oil on alternate days. The results of this experiment are shown in Fig. 4. Two of these ewes, Y.380 and X.300, aborted after receiving a total of 120 and 280 mg. hexoestrol respectively. M.907 gave birth to a normal ewe lamb after receiving two doses totalling 80 mg. hexoestrol. Administration of hexoestrol to M.891 was discontinued, after 4 injections of 40 mg. each, to determine whether she would then regain her appetite. As may be seen in Fig. 4, an immediate recovery of normal appetite occurred. Ewe M.884 was evidently abnormal when the experiment commenced and, probably for this reason, was more seriously affected by the injections. At autopsy a partially mummified foetus was found in the uterus. Loss of appetite was particularly noticeable in the sheep receiving 40 mg. doses and it would appear that this dose rate is only just above the threshold of tolerance for pregnant ewes such as these.

Group 3.

Five ewes, about three months pregnant, were penned together and treated with progressively increasing daily doses of 5, 10 and 20 mg. of hexoestrol. The food consumption, which is recorded as the average for each day, and the dose rates are given in Fig. 5. On the thirty-

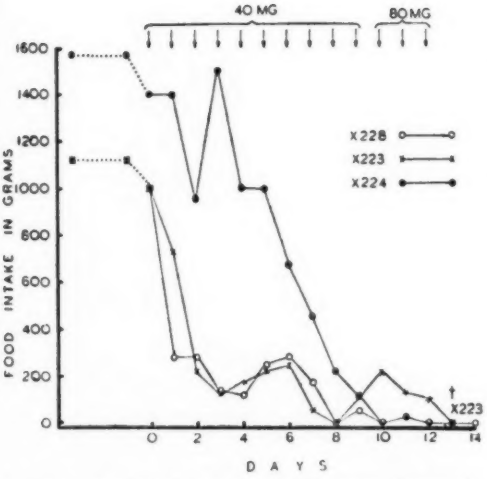

Fig. 3. Daily food intake of three ewes (Group 1) treated with hexoestrol by daily injections. The initial dose rate of 40 mg. hexoestrol per sheep was increased to 80 mg. on the tenth day as shown. The first two points on each curve, joined by dotted lines, represent the level of mean daily food intake for the previous three weeks. Sheep X.223 died on the thirteenth day, as shown by the cross.

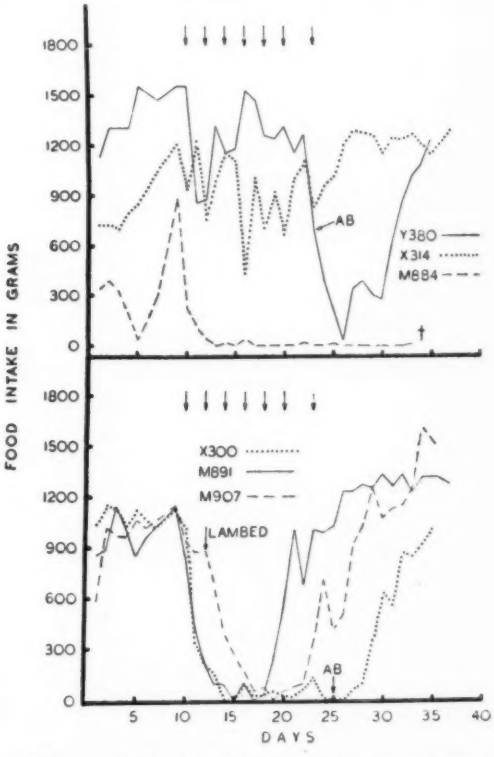

Fig. 4. Daily food intake of six pregnant ewes (Group 2), which received hexoestrol by injection on alternate days. Three sheep, Y.380, X.314 and M.884 (top), received 20 mg. hexoestrol per dose, and the other three, X.300, M.891 and M.907 (bottom), 40 mg. per dose. Administration of hexoestrol to sheep M.891 was discontinued after the fourth injection, and to M.907 after the second injection. AB signifies that the sheep aborted on the day shown.

second day, two ewes were removed from the group. One of the remaining sheep died in dystocia on the sixty-first day. The other two went down and were killed *in extremis* after forty-two and forty-seven days respectively. The daily food intake became very erratic after the hexoestrol injections were commenced, but showed no general tendency to decline until about the thirtieth day. Even towards the end of the experiment the food intake did not decline to the degree observed in the sheep in other groups.

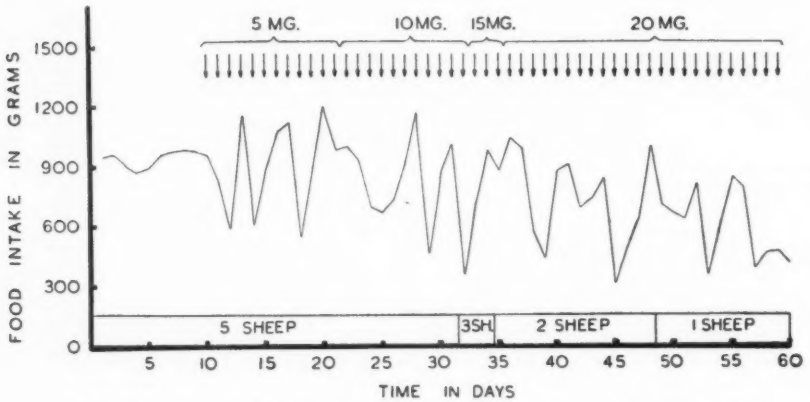

Fig. 5. The mean daily food intake of five pregnant ewes (Group 3) which received hexoestrol as indicated by the arrows. The number of sheep decreased, as a result of death or withdrawal from the group, as shown.

DISCUSSION.

Hexoestrol consistently reduced the food intake of sheep, but there seemed to be no clear correlation between the size of the dose and the degree of its effect. It appeared that the frequency of administration was more important provided the dose was sufficiently high. This was consistent with the transient effect of this oestrogen upon the appetite. From this point of view the effect on the appetite differed from the oestrogenic action of hexoestrol. In order to produce lasting inappetence it was necessary to administer the hormone no less frequently than every two or three days.

Since the completion of this work, Bennetts (1946) has recorded that stilboestrol produced some anorexia in Merino wethers, and Cameron, Guthrie and Carmichael (1946) have observed a reduced food consumption in rats given oestradiol in the food. It thus appears that the natural oestrogens as well as stilboestrol and hexoestrol affect the appetite.

SUMMARY.

Twenty-four ewes were treated with hexoestrol which greatly depressed the appetite.

The effect was brief, and continued inappetance was produced only when hexoestrol was administered every two or three days.

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THE INFLUENCE OF FRUCTOSE ON THE UTILIZATION OF GLUCOSE BY ISOLATED MUSCLE

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(Accepted for publication 18th June, 1947.)

In previous experiments (Corkill and Nelson, 1940) we demonstrated that fructose markedly increased the peripheral utilization of glucose and furthermore that under the stated experimental conditions was not utilized by muscle tissue. These experiments were carried out in spinal eviscerated cats[1] receiving a continuous intravenous infusion of glucose and in control tests the decrease in blood glucose following insulin was observed over a two-hour period. In contrast to these experiments, in those where both glucose and fructose were infused there was an approximately 100 p.c. increase in the amount of glucose disappearing from the circulation. No observations were made on O_2 consumption or muscle glycogen and accordingly it was thought worthwhile to seek information along these lines by means of the rats' diaphragm technique as described by Gemmill (1941). The latter observed that insulin acting on the diaphragm muscle could promote a deposition of glycogen, but no increased oxygen consumption occurred. The glucose that disappeared under the influence of insulin could be completely accounted for by O_2 consumption and glycogen deposition.

The following experiments are concerned with the influence of fructose on O_2 consumption of, and glycogen formation in, muscle strips in contact with insulin. The technique and chemical methods described in a previous paper were followed (Nelson, 1944).

Fructose was estimated by the modified method of Roe (1934).

RESULTS.

The Effect of Fructose on the O_2 Consumption of Isolated Muscle.

According to Gemmill *et al.*, glucose concentrations varying from 0 to 500 mg. p.c. have no effect on the O_2 consumption which is approximately 1·00 mm.^{3} per mg. wet weight per hour. In the following experiments the diaphragm was divided into two parts one of which (a) was incubated with 0·2 p.c. glucose, whilst the other (b) was in contact with 0·2 p.c. glucose + 0·05 p.c. fructose. The figures in Table 1 show O_2 consumption (mm.^{3}) per mg. wet weight per hour.

TABLE 1.

Experiment No.	1	2	3	4	5	6	7	8	9	10	Mean
0·2 p.c. glucose (a)	1·02	1·05	1·01	1·00	0·96	1·00	0·95	1·04	1·01	1·03	1·01
0·2 p.c. glucose + 0·05 p.c. fructose (b)	0·97	1·03	1·01	1·04	1·04	1·05	0·98	1·02	1·04	1·01	1·02

From these results it would appear that fructose has no effect on O_2 consumption.

The Effect of Fructose on the Deposition of Glycogen.

Before proceeding to study the effect of fructose on glucose utilization by insulin it was necessary to carry out some preliminary control experiments. From Gemmill's results it appears that with glucose concentration over 300 mg. p.c., in the absence of insulin, the glucose that

[1] In addition to the intestines, the kidneys were removed.

TABLE 2.

The effect of glucose concentration and addition of fructose on glucose utilization and glycogen deposition without insulin.

The diaphragm was divided into three portions: (*a*), (*b*) and (*c*). (*a*) was incubated with 0·2 p.c. glucose; (*b*) with 0·25 p.c. glucose, and (*c*) with 0·2 p.c. glucose + 0·05 p.c. fructose.

Glucose loss p.c.			Glycogen p.c.			Fructose concentration p.c.	
0·2 p.c. glucose.	0·25 p.c. glucose.	0·2 p.c. glucose + 0·05 p.c. fructose.	0·2 p.c. glucose.	0·25 p.c. glucose.	0·2 p.c. glucose + 0·05 p.c. fructose.	Initial.	Final.
(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)		
0·45	0·47	0·43	0·34	0·38	0·36	·048	·046
0·52	0·58	0·60	0·46	0·44	0·49	·048	·044
0·48	0·46	0·52	0·52	0·48	0·44	·051	·051
0·45	0·47	0·41	0·26	0·30	0·30	·051	·055
0·47	0·47	0·39	0·21	0·25	0·24	·049	·047
0·44	0·45	0·42	0·24	0·23	0·21	·049	·049
0·41	0·46	0·44	0·25	0·27	0·21	·050	·052
0·51	0·49	0·47	0·21	0·20	0·25	·050	·050
0·48	0·50	0·45	0·28	0·26	0·31	·052	·049
0·40	0·46	0·40	0·34	0·34	0·31	·052	·051
0·42	0·42	0·38	0·27	0·25	0·29	·049	·051
0·43	0·48	0·43	0·31	0·32	0·26	·049	·050
$0·45_5$	$0·47_6$	$0·44_5$	$0·30_8$	$0·31_0$	$0·30_6$	Mean	

TABLE 3.

Effect of fructose on glycogen deposition by insulin.

The diaphragm was divided into three parts: (*a*) incubated with 0·2 p.c. glucose, (*b*) incubated with 0·2 p.c. glucose + insulin (2 units), (*c*) incubated with 0·2 p.c. glucose + 0·05 p.c. fructose and insulin (2 units).

	Glucose loss p.c.			Increase in glucose loss p.c.		Glycogen p.c.			Increase in glycogen p.c.			Fructose concentration.	
	(*a*)	(*b*)	(*c*)			(*a*)	(*b*)	(*c*)					
No.	Glucose.	Glucose + insulin.	Glucose + fructose + insulin.	With insulin.	With fructose and insulin.	Glucose.	Glucose and insulin.	Glucose + fructose + insulin.	With insulin.	With fructose and insulin.	Difference.	Before incubation.	After incubation.
1	0·49	0·73	0·82	0·24	0·33	0·39	0·60	0·72	0·21	0·33	+0·12	0·51	0·51
2	0·44	0·70	0·81	0·26	0·37	0·45	0·70	0·80	0·25	0·35	+0·10	0·51	0·53
3	0·46	0·68	0·77	0·22	0·31	0·43	0·63	0·73	0·20	0·30	+0·10	0·51	0·52
4	0·39	0·60	0·69	0·21	0·30	0·53	0·63	0·95	0·10	0·42	+0·32	0·50	0·49
5	0·41	0·53	0·80	0·12	0·39	0·36	0·47	0·74	0·11	0·38	+0·27	0·50	0·47
6	0·37	0·60	0·66	0·23	0·29	0·44	0·65	0·72	0·21	0·28	+0·07	0·50	0·45
7	0·49	0·68	0·77	0·19	0·28	0·28	0·50	0·57	0·22	0·29	+0·07	0·49	0·51
8	0·56	0·85	0·85	0·29	0·29	0·56	0·87	0·90	0·31	0·34	+0·03	0·49	0·50
9	0·47	0·69	0·73	0·22	0·26	0·50	0·68	0·75	0·18	0·25	+0·07	0·49	0·49
10	0·54	0·91	0·90	0·37	0·36	0·29	0·69	0·67	0·40	0·38	−0·02	not estimated	
11	0·51	0·71	0·75	0·20	0·24	0·24	0·42	0·47	0·18	0·23	+0·05	not estimated	
12	0·42	0·65	0·56	0·23	0·14	0·42	0·68	0·55	0·26	0·13	−0·13	not estimated	
13	0·58	0·74	0·85	0·16	0·27	0·41	0·61	0·71	0·20	0·30	+0·10	0·52	0·50
14	0·54	0·80	0·95	0·26	0·41	0·55	0·82	0·97	0·27	0·42	+0·15	0·52	0·51
15	0·47	0·65	0·83	0·18	0·36	0·44	0·63	0·82	0·19	0·38	+0·19	0·52	0·56
16	0·42	0·80	0·79	0·38	0·37	0·55	0·92	0·93	0·37	0·38	+0·01	0·52	0·52
Mean	$0·47_3$	$0·70_8$	$0·78_3$	$0·23_5$	$0·31_1$	$0·42_8$	$0·65_6$	$0·75_0$	$0·22_9$	$0·32_3$	$+0·09_4$	$0·50_6$	$0·50_4$

$t = 2·4$
$p = 0·02$,
when $t = 2·6$
$p = 0·05$,
when $t = 2·13$

disappears cannot be fully accounted for by oxidation. Further, when insulin is added the glycogen deposition, if the initial glucose concentration is above 200 mg. p.c., does not reconcile with the glucose loss. Since in our experiments the total hexose concentration was 250 mg. p.c., balances were made with this concentration of glucose and glucose + fructose.

These results of our experiments, with and without insulin, are shown in Tables 2 and 3.

SUMMARY.

Glucose disappearing without insulin is unaffected by the addition of fructose.

Fructose in the absence of insulin does not cause deposition or diminution of muscle glycogen.

Fructose without the intervention of insulin is not utilized under the above experimental conditions.

Variation of hexose concentration within the range of these experiments does not alter the muscle glycogen level.

Fructose causes a significant increase in the rate of disappearance of glucose under the influence of insulin. The increase in glucose utilization can be accounted for by glycogen deposition.

Acknowledgments: The Authors wish to express their thanks to Mr. A. Doutch for a statistical analysis of results presented in Table 3. Thanks are also due to the Director of the Commonwealth Serum Laboratories for supplies of crystalline insulin.

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AN OPTICAL METHOD FOR MEASURING SYSTOLIC BLOOD-PRESSURE IN THE INTACT RAT

by F. B. BYROM[1]

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(Accepted for publication 1st August, 1947.)

Of the various methods available for measuring blood pressure in the intact rat, the plethysmographic technique (Byrom and Wilson, 1938; Williams, Harrison and Grollman, 1939) has probably been most widely used in recent years. While satisfactory results have been obtained by this method a water plethysmograph on the tail is a rigid, cumbersome device, requiring immobilization of the rat. In a search for a more flexible alternative, many devices for detecting the arterial pulse in the tail have been tried by the writer and discarded. The method described in this paper was finally adopted as the simplest and most satisfactory.

The method is adapted from the familiar air oscillometer as used on man and on the rabbit's ear (Landis, Montgomery and Sparkman, 1938). The pulsations of the caudal artery are conducted from an air cuff encircling the tail through a light, narrow rubber tube to a sensitive rubber tambour carrying a minute mirror which deflects a beam of light. The oscillations of the optical lever are observed on a ground-glass screen. A second air pressure cuff on the root of the tail is used to occlude the artery.

EXPERIMENTAL.

The apparatus is illustrated in Figs. 1 and 2 and is constructed as follows:

The *occluding cuff* is identical with that described by Byrom and Wilson (1938). Since some difficulty may be encountered in making neat cylindrical sleeves out of thin sheet latex, some amplification of the original description is desirable. A piece of thin, best-quality latex, about 5 cm. square is moistened with water and wrapped evenly round a glass rod of appropriate diameter, e.g. 9 mm., leaving a free rectangular flap about 1·5 cm. broad. The wrapped rod is carefully gripped in a paper clamp of the kind shown in Fig. 3 and the protruding flap is reflected evenly over one jaw of the clamp and tacked to the jaw with a speck of rubber cement at each free corner. The strip of latex exposed between the jaws is cleaned, dried and then evenly smeared with rubber solution with a single sweep of the finger. The adjacent part of the flap is similarly treated. The smeared surfaces immediately swell up into folds and wrinkles, but if left untouched for 30 minutes or more they resume their original form. The flap may now be freed and pressed down to make a neat seam, and it only remains to trim off excess latex from the outer and inner surface of the sleeve. The further construction of the cuff should present no difficulty.

Shuler, Kuppelman and Hamilton (1944) have studied the effect of cuffs of various widths on readings obtained by the plethysmographic method of Williams, Harrison and Grollman (1939), using the accurate Hamilton manometer as a standard of comparison. They found that widely different results could be obtained by altering the effective width of the cuff. With 16 mm. cuffs the plethysmographic readings at various pressures ran parallel with the standard systolic values, but were lower by about 36 mm. Hg. Shuler, Kuppelman and Hamilton, therefore, suggested that cuffs of 16 mm. should be used in future and that a correction of 36 mm. should be applied to the results. Byrom and Wilson (1937) had arbitrarily chosen a 15 mm. cuff in their plethysmographic technique and in view of the above findings the same width has been adopted in the present technique. In the writer's opinion less confusion is likely to be caused if results are recorded, as in the past, as actual readings, the reader being left to apply a correction if he so desires.

[1] The expenses of the research have been defrayed by grants from the National Health and Medical Research Council.

The cuff is connected through a convenient length of narrow (3 mm. outer diameter) rubber tubing and a 3-way tap to a mercury manometer and a sphygmomanometer bulb and release valve. The *oscillometer cuff* (Fig. 2) is similar to the occluding cuff, but the sleeve should be longer (20–25 mm.) and the celluloid outer case should be brought as near to the inner sheath as possible, in order to minimise dead space in the oscillometer system. The cuff is made airtight by painting with celluloid cement made by dissolving scrap celluloid (X-ray film) in acetone. It is connected to the oscillometer unit by a length of rubber tubing not exceeding 25 cm. long by 3 mm. diameter, the bore of which should not exceed 1·5 mm. A 3-way tap of similar bore is interposed near the oscillometer. The *tambour* consists of thin latex tied over the end of a short length of glass tubing or a perspex cup, 1 cm. or less in diameter. The membrane should be flat but not stretched. The dead space in the tambour should be kept as small as possible and the connecting tube should be free from constrictions and sharp bends.

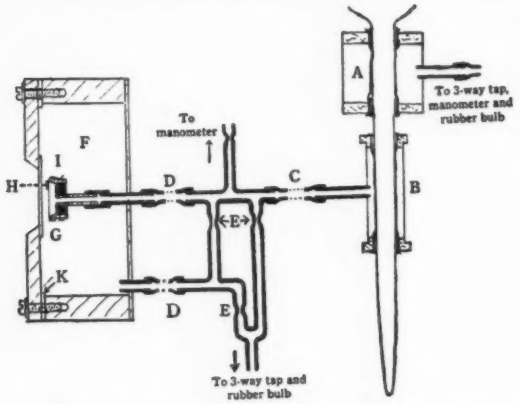

Fig. 1. Oscillometer Assembly. A, Occluding cuff encircling root of rat's tail; B, Oscillometer cuff; C, Connecting rubber tubing; D, Shorter lengths (8 cm.) of rubber tubing; E, Adjustable constrictions; F, Pressure equalization chamber; G, Tambour; H, Oscillometer mirror; I, Coverslip window; K, Rubber gasket.

The *pressure equalization system* (Fig. 1). A slight positive pressure (10–50 mm. Hg.) is desirable, though not always necessary, in the oscillometer cuff, to ensure good contact with the tail, and also to keep the cuff in position. It is, therefore, necessary to enclose the tambour in an air-tight chamber with suitable communicating tubes for inflation and pressure equalization. An auxiliary mercury manometer is also desirable to indicate the pressure in the system. The system is essentially that described by Landis, Montgomery and Sparkman (1938) for use with the rabbit's ear. The present model has been constructed of metal, using narrow-bore brass tubing and adjustable needle valves. Perspex, glass and rubber tubing with screw clips suggest themselves as suitable alternatives. The tambour should be accessible for replacement of the membrane.

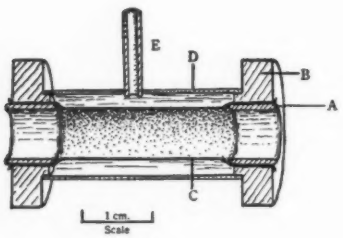

Fig. 2.

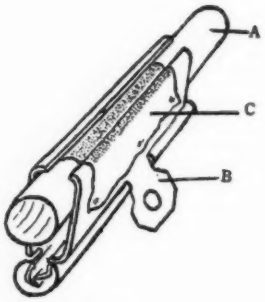

Fig. 3.

Fig. 2. Section of Oscillometer Cuff. A, Glass or perspex collar; B, Cork end-discs; C, Latex sheath; D, Celluloid case; E, Air inlet tube.

Fig. 3. Construction of Latex Sheath (see text). The latex has been wrapped round the glass rod or tube A, and fixed in the paper clamp B. The flap of latex C, has been reflected over one jaw of the clamp. The stippled areas are smeared with rubber solution and left until the solvent has evaporated, when they may be pressed together.

The *optical system* need not be elaborate. A 35 watt car headlamp bulb, a pin hole or slit and a simple convex lens of long focal length should be adequate. In the present apparatus the writer uses a 10 volt, 70 watt "excitor" lamp, as used in sound projection units, operated by a mains transformer.

The *mirror* is cut with a sharp diamond from an optically flat piece of back-silvered cover slip. It should not exceed 2 by 1·5 mm. in size. It is attached to the periphery of the tambour by a speck of gum. The optical lever should be as long as possible, preferably not less than four metres. Large auxiliary fixed mirrors are used to reflect the beam on to a ground-glass screen near the operator.

The *anaesthetizing box.* Any warmed box or jar may be used for this purpose, but to save time in dealing with large numbers of rats a more elaborate anaesthetizing cabinet has been constructed. This consists of a tinplate box insulated with asbestos and divided into three equal rectangular compartments, each measuring 8 by 4 by 4 inches, arranged side by side. Each compartment has a hinged perspex lid with a central hole, 1¾ inches diameter, covered with metal (fly screen) gauze and surmounted by a metal pill box to hold a swab soaked with ether. At the front end of each compartment is a hinged-door covering a hole, 1 inch square, for the tail. Wire-meshed trays for excreta are provided. A row of flat nichrome heating elements, wired in series, is bolted to the under surface of the box. These dissipate about 120 watts on 240 volts and are controlled by an adjustable bimetallic (Sunvic) thermostat in the bottom of the central compartment.

When the oscillometer has been assembled it should be tested for sensitivity by lightly pressing the pad of a finger against one end of the oscillometer cuff, the other end having been occluded. If the hand is reasonably warm distinct oscillations from the digital arteries, with an amplitude of at least 1 cm. should be visible on the screen.

Measurement of Systolic Pressure.

With anaesthesia. For reasons which will be described the writer prefers to use ether anaesthesia in measuring blood pressure. Ether tends to depress the rat's blood pressure slightly, if correctly administered, and markedly if anaesthesia becomes too deep, and negative errors are preferable in most studies of blood pressure. Positive errors with ether are usually due to one of two easily recognizable causes, namely partial anaesthesia and early respiratory obstruction by mucus. Partial anaesthesia is indicated by voluntary movements of the whiskers, retention of postural muscular tone, and a positive corneal reflex. Respiratory obstruction can be relieved by aspiration through a suitable cannula. Persistent obstruction causes a pronounced fall in pressure.

Warming of the rat is not always necessary if ether is used, for its objective—to abolish spontaneous variations in peripheral vascular tone—is partly achieved by the anaesthetic. In practice, however, it is found that warming the anaesthetizing box to 38° to 40° C. gives clearer readings, especially with sick rats, where the pulse may be very feeble, and entails no risk of false high readings unless the measurement is unduly delayed.

The rat is placed in the anaesthetizing chamber and a swab soaked in ether is put in the pill box. Good oscillations will not usually be obtained until the ether and the warmth have taken effect and about three minutes should elapse before a reading is attempted. The lid is then propped open and the tail is drawn through the special opening and threaded through the two cuffs. When the oscillometer cuff is inflated to a pressure of 10–50 mm. Hg. oscillations with a frequency of 250–300 cycles per minute and an amplitude of 5–50 mm. should be visible on the screen. When they appear the occiuding cuff may be steadily inflated until the oscillations diminish and then disappear, when the manometer is read. The pressure is now gradually released and a second more careful reading is taken as soon as continuous oscillations appear. This second reading is always lower by 10–20 mm. Hg. than the first. A similar but greater discrepancy has been reported by Griffith and Farris (1942) using an earlier indirect technique. The lower reading is arbitrarily taken as the systolic pressure, partly because deflation of the cuff can be more finely controlled than inflation and partly because the end point in the plethysmographic technique has necessarily been measured during deflation of the cuff. The work of Shuler, Kuppelman and Hamilton (1944), nevertheless, suggests that the higher first reading is probably nearer to, though still lower than the actual systolic pressure. The oscillometer cuff can be used as a plethysmograph if means are provided to close the communicating tubes in the instrument, but the pulse oscillations provide a much more satisfactory end point. As soon as the reading has been checked the cuffs are deflated through 3-way taps, the rat is removed and another one is placed in the compartment. Meanwhile the rat in the next compartment will be anaesthetized ready for measurement and by using the three compartments in succession large numbers of animals can be dealt with in a short time.

Without anaesthesia. The decision to avoid anaesthesia should not be lightly taken. It is true that after preliminary training many rats will give consistent readings, but even with trained rats meticulous care must be taken to avoid rough handling, injections, and unusual noises, voices and smells (Kempf and Page, 1942). Quite minor lapses, often not obvious to the operator, will excite the rat and raise its blood pressure by 40 mm. Hg. or more. Warming

of the rat is also necessary but overheating is a common source of gross positive errors (Proskauer, Newman and Graef, 1945). These writers also examined the effect of various kinds of rat holders on the pressure readings and found that holders made of sheet metal, as distinct from metal gauze gave rise to excessively high readings. Similar results were obtained by the present writer when warmed metal tubes were used as holders. Finally, in some unanaesthetized rats, especially when excited or struggling, rhythmical variations in pressure of the order of 40 mm. Hg. and with a frequency of about 20 cycles a minute are observed. These appear to be Sigmund-Mayer waves and are probably responsible for the occasional indefinite end points encountered when using the plethysmographic technique. They are rarely conspicuous in anaesthetized rats.

For these reasons, if an experiment requires that anaesthesia must be omitted, the operator should take particular care to exclude these several sources of positive error, and this will add greatly to the time and labour involved.

Normal Range of Systolic Pressure.

Using ether anaesthesia as described above, readings have been made on 107 male and 32 female Wistar rats ranging from 30 to 310 gm. in weight. Readings ranged from 80–160 mm. Hg. with a mean of 115·6 mm. and a standard deviation of 12·76. Only one reading over 144 mm. and three readings below 90 mm. were obtained. The mean is about 10 mm. higher than that reported by Byrom and Wilson (1938). The use of a warm anaesthetizing chamber probably accounts for this difference. Slightly lower readings were obtained in infant rats, possibly owing to the use of comparatively long occluding cuffs.

Advantages of the technique. Although the assembled apparatus is rather bulky and a darkened room is necessary the oscilloscopic technique has several advantages over most other indirect methods. The apparatus is simple and inexpensive, the oscillations which mark the end point are unmistakable, measurements are easily and quickly made, the light weight of the cuffs (about 2·5 gm. each) and the connecting tubes causes minimal embarrassment to the rat and obviates rigid restraint of the animal, the optical lever automatically readjusts itself if the animal should move and the method is sensitive enough to give quite clear readings with rats weighing as little as 30–40 gm. The method is obviously applicable, with suitable modifications, to the limbs or tails of larger animals.

Diastolic Pressure.

By using a single cuff, measurement of diastolic pressure should be practicable, but the more refined technique and complex apparatus of Landis, Montgomery and Sparkman (1938) would be necessary. For systolic measurements a single cuff merely complicates the technique and gives a less clear-cut end point, since oscillations can still be perceived after the pressure in the cuff has far exceeded the systolic level.

SUMMARY.

Systolic blood pressure is measured in the rat by means of two air cuffs encircling the tail. The proximal cuff is used to occlude the artery. From the distal cuff the arterial pulse waves are transmitted through light flexible tubing to a sensitive tambour carrying a mirror which operates an optical lever.

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ON THE DECOMPOSITION OF METHAEMOGLOBIN BY HYDROGEN PEROXIDE

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In general the derivatives of the blood pigment that contain native protein are not very susceptible to attack by hydrogen peroxide. The accompanying catalase, or their own inherent catalatic power, by decomposing the peroxide protects them from its action. Ferrous derivatives are not, to any great extent, oxidized to ferric. Three exceptions, however, have been recorded.

Barkan and Schales (1938) showed that hydrogen peroxide, in the presence of potassium cyanide, acts on oxyhaemoglobin to form *inter alia* a greenish pigment with a strong absorption band in the red region of the spectrum. They named this pigment "pseudo-haemoglobin." Lemberg (1941) identified it as a cyanide compound of choleglobin, the protein of which was mainly denatured during its formation. Its constitution, like that of choleglobin, has yet to be established. Lemberg (1941) also showed that, in the absence of oxygen, hydrogen peroxide converts haemoglobin to choleglobin to the extent of 16 p.c. of the amount of the pigment present, a yield about one-half of that obtained by the incubation of oxyhaemoglobin with ascorbic acid. Brooks (1937) mentioned that in slightly acid solution and in the presence of sodium hyposulphite and 0·05 M sodium nitrite oxygen could convert nitric oxide haemoglobin to a greenish pigment whose oxidized form had an absorption band at 615 mμ and its reduced form at 618 mμ. Nothing further was said about its properties or constitution. Since some hydrogen peroxide was probably produced during the reaction the pigment obtained may have been a mixture derived partly from oxidative disruption of the haemoglobin and partly from its decomposition by hydrogen peroxide.

The experiments described in this paper are attempts to gain further insight into the class of reactions just outlined. They have now to be laid aside for a considerable time and are set down in hope that they will assist some other worker interested in the chemistry of the blood pigment.

EXPERIMENTAL.

Experiments on the action of hydrogen peroxide and sodium nitrite on oxyhaemoglobin yielded unsatisfactory mixtures. They showed, however, that only in weakly acid solution was there any oxidative disruption of the prosthetic group. In neutral or alkaline solution the products of the action of sodium nitrite on oxyhaemoglobin or methaemoglobin appear to exhibit the normal catalatic activity of solutions of the blood-pigment. In acid solution this is inhibited and, on the addition of hydrogen peroxide, a greenish solution results.

The course of the reaction was studied in the following manner; 5 ml. of a 15 p.c. solution of autoxidation horse methaemoglobin at pH 5·3 were mixed with an equal volume of 1 p.c. sodium nitrite at the temperature of the laboratory; 1 ml. of approximately 1 p.c. hydrogen peroxide was added. Samples were removed after one minute, three minutes and each third minute thereafter for a quarter of an hour. These were at once dropped into tubes containing 4 p.c. sodium hydroxide and a little pyridine. These alkaline solutions were reduced with sodium hyposulphite and examined with a direct-vision spectroscope fitted with a wave-length scale and also, when suitable, with a Hartridge spectrometer. At first, in addition to the bands of pyridine proto-haemochromogen, there was seen a vague band in the orange-red region at about 606 mμ. Later this band became clearer and shifted to about 611–613 mμ and a second band at 580 mμ was seen. Later this second band faded and there was only a vague band at

610 mμ. When a sample was reduced in dilute ammonia without the denaturant effects of caustic alkali or pyridine the band at 580 mμ was not seen but only the band at 611 mμ. When the sample was reduced in sodium hydroxide without pyridine the protein, denatured by the caustic alkali induced the formation of a band at 580 mμ but it was less intense than when pyridine was present. Thus two pigments are apparently formed. The band at 611 mμ is not intensified appreciably by carbon monoxide, as is the case with the bands of choleglobin, pseudohaemoglobin and cruoralbin. The pigment showing the band at 611 mμ has not been detached from the protein but is lost or destroyed during the preparation of the prosthetic group responsible for the band at 580 mμ. For this preparation the following conditions were found suitable: 50 ml. of a concentrated solution of autoxidation horse methaemoglobin or cyanmethaemoglobin (without much excess of cyanide) were adjusted to about pH 5·5 and cooled to 0° C. in a one litre bottle; 25 ml. of 1 p.c. sodium nitrite also cooled to 0° C. were added and, immediately after mixing, 1 ml. of 100 volume hydrogen peroxide diluted with 25 ml. ice-cold water was poured in. The liquid quickly turned green and was kept at 0° C. till observation with a direct-vision spectroscope showed that the spectrum had changed to one showing a single band in the orange region. The prosthetic group was then detached with 100 ml. glacial acetic acid, 250 ml. amyl alcohol and 50 ml. saturated ammonium sulphate solution, added in this order. After removal of the protein by filtration, the alcoholic layer was repeatedly washed with water and the pigment extracted thence with aqueous ammonia. After filtration of the ammoniacal solution the pigment was precipitated with dilute hydrochloric acid, washed and dried *in vacuo*.

TABLE 1.

Wavelengths in mμ *of the absorption bands in the visible spectra of various substances mentioned in the text.*

Substance.	Derivative.	Solvent.	
Green pigment from Methb. $NaNO_2$ H_2O_2	ferric, native protein	water	592
	ferrous, native protein, early	water	620
	ferrous, native protein, later	water	611
	ferrous, native protein, CO	water	616
	pyridine hchr.		
	protein present	NaOH	613, 580
	denatured globin hchr.	NaOH	611
Free prosthetic group of above	ferric	pyridine	581
	ferrous	dil. NH_3	620 (vague)
	pyridine hchr.	NaOH	582, 552, 521
	NH_3 hchr.	conc. NH_3	587, 555, 522
Porphyrin of above	hydrochloride	conc. HCl	615, 564
	hydrochloride	$CHCl_3$	621, 569
	hydrochloride	glacial acetic acid	618, 568
	neutral	pyridine, $CHCl_3$	648, 590, 561, 516
	Zn complex	glacial acetic acid	610, 544
	Cu complex	glacial acetic acid	597, 546
	Ni complex	glacial acetic acid	586, 556
	Co complex	glacial acetic acid	595, 545
Bile pigment formed by second method	hydrochloride	dil. HCl	652

The solution of this prosthetic group in pyridine is of a bright green colour; its spectrum has a strong band at 581 mμ. When reduced in aqueous pyridine there are seen two bands near to, but not identical with, those of pyridine proto-haemochromogen and a third band at 586 mμ. Reduced in concentrated ammonia the spectrum is similar with bands at 587, 555 and 522 mμ.

It was at first thought that the two bands in the green were those of proto-haemochromogen but the following evidence casts some doubt on this view. When dissolved in dilute aqueous ammonia the pigment is not adsorbed on a column of calcium or magnesium carbonate, both of which adsorb proto-haematin very strongly in a narrow zone at the top of the column. When proto-haematin is dissolved in dry pyridine and gently shaken with a bubble of mercury the pigment is at once reduced to pyridine proto-haemochromogen. No such reduction of this pigment could be induced by this means. On reduction with formic acid and iron or stannous chloride and

alcoholic hydrogen chloride the pigment was decomposed to a tawny product with the formation of but an insignificant trace of protoporphyrin. No clear spectroscopic evidence was obtained of the pigment's combining with carbon monoxide or of forming a compound analogous to oxyhaemoglobin.

When the pigment was dissolved in concentrated sulphuric acid it was converted into a green porphyrin with the absorption bands nearer the infra-red than those of proto-porphyrin.

A better method of preparation of the porphyrin, however, was found to be to heat the pigment cautiously in formic acid containing a lot of oxalic acid with iron powder. It was soluble in concentrated hydrochloric acid but was precipitated on the addition of water. Solutions of a few metal-complexes of the porphyrin were prepared for spectroscopy by heating the porphyrin with the appropriate metal acetate in acetic acid.

A different pigment was prepared by the following procedure. To 50 ml. of a solution of cyan-methaemoglobin adjusted to pH 6 there were added successively 50 ml. of a solution containing 2 p.c. hydrocyanic acid (prepared by adjusting potassium cyanide solution to pH 6 with acetic acid) 2·5 ml. of 10 p.c. sodium nitrite solution, and 0·25 ml. of 100 volume hydrogen peroxide in 25 ml. water. No catalatic decomposition of the peroxide was seen nor was there any change in the visible spectrum. When, however, after about two minutes a sample was heated with formic acid and iron the spectrum showed, in addition to weak bands of acid proto-porphyrin, a strong band in the red at 652 mμ. The reaction was carried out at 0° C. and after 10 minutes, on account of the lower temperature, the prosthetic group was detached with amyl alcohol and acetic acid as in the case of the first pigment. It showed the band at 652 mμ on reduction with formic acid and iron. If too little cyanide or too much peroxide were used or the reaction time was prolonged this substance was not detected and the chief resultant was the first pigment, whose pyridine parahaematin shows a band at 581 mμ.

Another pigment was prepared from methaemoglobin in the following way: In a test tube were placed, in this order, 1 ml. of 2 p.c. methaemoglobin of pH 5·2–5·6, 2 ml. 4 p.c. sodium fluoride, 0·5 ml. 1 p.c. sodium nitrite and 0·8 ml. 0·3 p.c. hydrogen peroxide. A single heavy band was seen at once at about 600–610 mμ. During the next minute a band developed in the far red region and in the meantime the first band gave place to two bands one at about 570 mμ and one at about 610 mμ. None of the bands was suitable for exact measurements with the Hartridge spectrometer. After another two or three minutes all the bands began to fade. When the far-red band was showing most clearly a sample was poured into sodium hydroxide solution containing pyridine. On reduction only a vague band at about 606 mμ was visible. After the bands in the oxidized form had faded another sample similarly reduced showed the bands at 580 and 611 mμ.

DISCUSSION.

The various decompositions of the blood pigment brought about by hydrogen peroxide yield products so diverse and have apparently so little in common that any discussion may well be regarded as of little value. For decomposition of the blood pigment by hydrogen peroxide it appears essential first to inhibit the catalatic activity of the solution of the blood pigment used. The small yield of choleglobin resulting from the action of hydrogen peroxide on haemoglobin, and the trifling yields from oxyhaemoglobin or methaemoglobin (Lemberg, 1941) may well be due to the catalatic powers of these solutions. Cyanide inhibits the catalatic power of solutions of haemoglobin and oxyhaemoglobin, but is far less active against that of cyan-methaemoglobin. Nitrite is inhibitory only in acid solution, not in neutral or alkaline solutions. Its inhibition in weak acid solution appears to be annulled on the addition of a little ammonia. Sodium azide behaves like potassium cyanide, but is unsatisfactory in that more side-reactions appear to occur.

The second point for consideration is the degree of reliance to be placed on spectroscopic appearances. There is a very strong tendency to rely on a spectrophotometric curve or a susceptibility determination to establish the constitution of a pigment without paying attention to its chemical reactions. The pyridine haemochromogen spectrum already noticed may owe two of its bands to contaminating proto-haem, but if it does it is strange that simple chemical tests do not confirm this view.

It is difficult to believe that the hydrogen peroxide has not had a profound effect on the cyan-methaemoglobin in the reactions leading to the formation of the

pigment that turns green on reduction with formic acid and iron but there is no change to be seen in the visible spectrum. The reaction was, in fact, first observed by chance, during a series of experiments designed for another purpose.

A third question arises as to the method of linkage to the blood pigment of the various reagents concerned in these reactions, cyanide to oxyhaemoglobin, nitrite to methaemoglobin, to cyan-methaemoglobin and to fluoride methaemoglobin.

The influence of hydrogen-ion concentration on these reactions is marked: it influences the relative speeds of the conversion of oxyhaemoglobin to pseudohaemoglobin and to cyanmethaemoglobin requiring the former to be prepared in an alkaline solution. It also controls the inhibition of catalatic activity by sodium nitrite. That cyanide should inhibit the catalatic activity of oxyhaemoglobin, with which it is not seen to combine, so much more strongly than that of methaemoglobin with which it is seen to combine is another awkward addition to the list of facts here surveyed. The inhibition of the catalatic activity of cyan-methaemoglobin by sodium nitrite in acid solution, and its restoration by the addition of a dilute alkali, are strongly suggestive of a type of dissociable combination in which the iron of the prosthetic group plays no part. Its available bonds are occupied by cyanide and globin.

Little can be said about the nature of the pigments described in this paper. The first resembles both the "haematins *a*" of "cytochrome *a*" and a pigment mentioned by Lemberg (1938). The spectroscopy of its porphyrin suggests that it may have a group attached to one of the methene carbons. The bright green substance with the band at 652 mμ formed by the reduction of the second pigment has properties that place it with such strongly basic bile pigments as biliverdin.

SUMMARY.

Three reactions between hydrogen peroxide and methaemoglobin are described. From one a haematin and porphyrin different from the protocompounds were prepared. From another, a green basic compound of the bile-pigment type was separated.

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SOME OBSERVATIONS ON FIBRINOLYSIN AND BETA TOXIN OF *STAPHYLOCOCCI*

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Coagulation of human or animal plasma by staphylococci and the subsequent digestion of such clots have been investigated by numerous workers. Dixon (1945), using living cultures as inocula, found that bactericidal concentrations of penicillin, while not inhibiting coagulation of plasma, did prevent subsequent fibrinolysis of the clot. These results suggested that either fibrinolysin was inhibited by penicillin or, if not, that inocula containing sufficient coagulase to produce a clot did not contain sufficient fibrinolysin to digest it.

Christie and Wilson (1941) examined a large number of strains of staphylococci of human and animal origin for fibrinolytic activity, using agar plates containing 12 p.c. heated plasma. They found that many coagulase-positive strains did not possess fibrinolytic activity on such plates. All such strains, the majority of which were of animal origin, proved on examination to be producers of β toxin. Christie, Graydon and Woods (1945) subsequently produced cell-free preparations of fibrinolysin and showed that the presence of β toxin had no inhibitory effect on the digestive action of the enzyme on fibrinogen in the test tube. They also found that even after neutralization with antitoxin of the β toxin present in a broth culture of a β toxigenic strain, no fibrinolysin could be demonstrated. These results indicate that the lack of fibrinolytic activity in β toxigenic strains is not due to inhibition of fibrinolysin by β toxin.

Examination of a number of strains of coagulase-positive staphylococci of human origin afforded an opportunity to make some observations on the effect of penicillin on fibrinolysin and on the relationship of fibrinolysin and β toxin.

METHODS.

Examination of cultures for the presence of β toxin was made by plating on sheep's blood agar (Bryce and Rountree, 1935). β toxin was prepared by growing suitable cultures in 0·15 p.c. glucose broth in 5 p.c. CO_2, centrifuging and adding 0·02 p.c. merthiolate to the supernatant fluid. The toxin was titrated against sheep's red blood cells. Fibrinolysin was prepared by growing cultures in broth containing 10 p.c. human plasma and filtering through a Seitz E. K. filter after four days' incubation at 37° C. Titrations of fibrinolysin were made by the method of Christie, Graydon and Woods (1945). Fibrinolysis by cultures was demonstrated on 12 p.c. heated human plasma agar plates (Christie and Wilson, 1941).

The Effect of Penicillin on Fibrinolysin.

If an actively fibrinolytic strain is inoculated on to the centre of a fibrinogen agar plate and incubated, a clear zone of lysis is produced around the inoculum. With continued incubation this zone extends and after five days may have a radius of 30–35 mm. It was found that if a block of agar containing no organisms was cut out from this cleared zone with aseptic precautions and placed on the surface of another fibrinogen plate, the fibrinolytic agent diffused out into the fresh medium and produced clearing. Fibrinogen agar plates were made in which penicillin was incorporated in concentrations of 1, 2·5 or 5 units per ml. Table 1 shows the size of the zones of clearing produced when agar blocks containing fibrinolysin were placed on the surface of these plates and incubated for 24 hours at 37° C.

TABLE 1.

Size of zones of fibrinolysis produced by agar blocks containing fibrinolysin on fibrinogen agar containing penicillin.

Penicillin concentration units/ml.	Size of agar block mm.	Size of zone of fibrinolysis mm.
0	6 × 14	15 × 19
1	7 × 15	15 × 21
2·5	6 × 10	8 × 12
5·0	8 × 14	10 × 15

The agar blocks used with the 2·5 and 5·0 units/ml. penicillin plates were thinner than those on the other plates and presumably contained less fibrinolysin. It is apparent that the presence of penicillin in the plates did not inhibit the fibrinolytic action. The conclusion was reached that penicillin has no action on fibrinolysin *per se*.

Confirmation of this was found when a Seitz broth filtrate was titrated for fibrinolysin by the method of Christie *et al.* (1945). The same end point was obtained with titrations containing a concentration of 10 units per ml. of penicillin in each tube and with titrations made in the absence of penicillin.

The inhibition of fibrinolysis obtained when broth cultures of staphylococci are titrated in plasma containing penicillin is, therefore, due to the destruction of the organisms by the penicillin before they have produced sufficient fibrinolysin to lyse the clot which they have formed.

Relationship of Fibrinolysin and β Toxin.

A series of 34 strains of coagulase-positive staphylococci of human origin was examined for fibrinolysin and β toxin. Thirty-one strains either digested fibrin clots in the test tube or caused clearing of fibrinogen agar. The three strains which showed no fibrinolytic activity were found to produce large amounts of β toxin, thus confirming the observations of Christie and Wilson (1941).

Inocula were made on the surface of fibrinogen agar plates of mixtures of a fibrinolytic strain and of β toxigenic strains. There was no inhibition of fibrinolysis in the presence of actively growing, β toxin-producing cells.

TABLE 2.

Fibrinolysis on fibrinogen agar with mixed inocula of fibrinolytic and β-toxigenic staphylococci.

Inoculum.	Toxin.	Fibrinolysis.
B.313	α	++++
3	β	—
B.313+3	α+β	++++
S.17	β	—
B.313+S.17	α+β	++++
211	β	—
B.313+211	α+β	++++

Citrated human plasma was heated at 56° C. for 30 minutes to precipitate the fibrinogen. Equal volumes of this heated plasma and of a preparation of β toxin were mixed, incubated at 37° C. for one hour and then centrifuged. The content of β toxin of the supernatant and of the original toxin were determined by titration with sheep's red blood cells in the usual manner.

The titres, allowing for dilution of the toxin, were identical, showing that there had been no absorption of β toxin on to fibrinogen in one hour at 37° C. The experiment was repeated, using both heated plasma and fibrinogen separated from plasma and resuspended in saline. These were mixed with equal volumes of β toxin and left in the ice chest for four days before titration of the toxin. Under these conditions there was no absorption or inactivation of β toxin by heated plasma or by fibrinogen. In carrying out such tests, it was found necessary to be certain that the human plasma used contained no β antitoxin. If β antitoxin was present, anomalous results were obtained.

It is apparent from these results, and from those of Christie, Graydon and Woods (1945), that the presence of β toxin in fibrinogen plates or in fibrinolytic titrations has no direct inhibitory effect on fibrinolysin. Nor does fibrinogen absorb β toxin and so become resistant to the action of fibrinolysin. It would seem that the inability of β toxigenic strains to lyse fibrin or fibrinogen is due to the fact that such strains do not produce fibrinolysin.

A strain of human origin, producing α toxin and actively fibrinolytic, was found on plating on sheep's blood agar to show evidence of the presence of β toxin-producing variants. These were revealed by intensification of the zones of α haemolysis around a few colonies, as described by Christie and North (1941). Such variants, when subcultured anaerobically, were found to produce β haemolysis on sheep's blood agar plates. They produced a large amount of α toxin and a comparatively small amount of β toxin. When tested for fibrinolysin on fibrinogen agar plates, they were completely devoid of activity, in contrast to the parent culture, which regularly produced wide zones of clearing.

The variation in this strain which resulted in the production of β toxin, therefore, also involved the loss of the ability to produce fibrinolysin. Whether this variation is due to a genetic change with which is linked the production of β toxin and the loss of fibrinolysin it is not possible to say. The site of production in the cell and the chemical nature of fibrinolysin and β toxin are not known and one can merely speculate as to whether the same group of metabolic components may produce fibrinolysin at one time and then by an alteration in their activity produce β toxin at another. It would seem, however, that the production of β toxin by a strain of staphylococcus, automatically excludes the production of fibrinolysin by that strain.

SUMMARY.

Fibrinolysis by cell-free preparations of staphylococcal fibrinolysin was not inhibited by penicillin.

Thirty-four coagulase-positive human staphylococci were examined for fibrinolysin and β toxin. Three strains which did not lyse fibrinogen produced β toxin. The presence of β toxin producers in mixed inocula with a fibrinolytic strain did not inhibit fibrinolysis on fibrinogen agar. There was no absorption of β toxin by heat precipitated fibrinogen.

The production of β-toxigenic, nonfibrinolytic variants in an α-toxigenic fibrinolytic strain is described and discussed.

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THE COMPARATIVE PHARMACOLOGY OF THE NOR-NICOTINES

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In their original communication on the isolation and identification of the alkaloid of *Duboisia Hopwoodii*, Hicks and LeMessurier (1935) reported the nicotine-like effect on the blood pressure which is obtained by the intravenous injection of 2 mg. into the intact rabbit. Hicks, v. Brücke and v. Hueber (1935) demonstrated that in the spinal cat, an intravenous injection of 2 mg. of the alkaloid, d-nor-nicotine, caused a pressor response equivalent to that produced by 0·1 mg. adrenaline, and established the rôle played by the suprarenals in this effect.

The present investigations began with a confirmation of the foregoing results from which point the quantitative study of the corresponding effects produced by laevo and dextro-nicotine and laevo and dextro nor-nicotine was developed.

EXPERIMENTAL.

Cats, anaesthetized with Dial (1 ml./kg. intraperitoneally) were used in these studies. The vagi were severed, artificial respiration established, and the viscera were removed. A cannula was tied into the vena cava at a point just anterior to the entry of the renal veins. 1 mg. atropin was injected and the preparation left for sixty minutes before insertion of the carotid cannula. The alkaloid solutions were injected into the vena cava in volumes of 1 ml. and washed in with a further 1 ml. of saline—all at 37° C.

Relation Between Dose of l-Nicotine and Pressor Effect and Adrenaline Secretion.

Injection of 0·2 mg. of l-nicotine caused a rise of blood pressure of 5 mm. Hg., 0·5 mg. a rise of 10 mm. Hg., whilst two successive injections of 1 mg. gave a pressor response of 17·5 mm. Hg. After extirpation of the suprarenal glands the pressor response to injection of 1 mg. fell to 6 mm. Hg.

Quantitative Relation of d-Nicotine, d-Nor-Nicotine and l-Nor-Nicotine in Terms of Pressor Response.

Injection of 0·5 mg. l-nicotine, after a preliminary slight fall gave a response of 59 mm. Hg.; 0·5 mg. l-nor-nicotine caused a rise of 16 mm. Hg. and was bracketed with a second dose of l-nicotine which gave almost the original pressor response, 53 mm. Hg.

0·5 mg. d-nor-nicotine now produced a rise of 14 mm. Hg.; 0·5 mg. d-nicotine caused a negligible rise of 6 mm. Hg., and was bracketed with a repeat injection of 0·5 mg. l-nicotine which again gave a response of the same order as the original—i.e., 40 mm. Hg.—followed after six minutes by a further dose of 0·5 mg. d-nicotine which again gave a rise of 6 mm. Hg.

After a lapse of one hour the assay was repeated, and although the sensitivity of the preparation had declined considerably, the quantitative results were substantially the same (vide protocols).

Following extirpation of the suprarenals the pressor response to 1 mg. each of the alkaloids was as follows: l-nicotine, 10 mm. Hg.; d-nicotine, 7 mm. Hg.; l-nor-nicotine, 8 mm. Hg.; d-nor-nicotine, 6 mm. Hg.

d-nicotine, therefore, would appear to have little or no effect upon the suprarenal glands.

[1] Under Grant from Council for Scientific and Industrial Research.

Effect of the Alkaloids on the Suprarenalectomized Animal.

For this experiment the glands were removed by lumbar incision and 1 mg. atropin injected by means of a cannula inserted in the femoral vein, the preparation being left for one hour before the assay. After injection of 0·25 mg. of the alkaloids the following typical results were obtained: l-nicotine, 20·5 mm. Hg.; d-nicotine, nil; l-nor-nicotine, 6·5 mm. Hg.; d-nor-nicotine, 12 mm. Hg. Repetition using 1 mg. of the alkaloids very slightly increased the effect (see protocols).

It is obvious that there is a quantitative relation between the dose of a member of this group of alkaloids and the quantity of adrenaline liberated from the suprarenal gland by the alkaloid. These results also indicate variations in the action of the different members of the group, d-nicotine in the concentration used having practically no effect upon the suprarenals.

Effects of the Nicotine Alkaloids upon Respiration.

Hicks, v. Brücke and v. Hueber (1935) using the method of van Esveldt found that after injection of 2·0 mg. of d-nor-nicotine into the decerebrate cat, 6 p.c. CO_2 failed to stimulate either the respiratory or the vasomotor centre. The latter recovered in 19 minutes, the former only after 67 minutes. It will be seen from the protocols that all four alkaloids in doses of 1–2 mg. influenced respiration, 3–5 mg. sufficing to paralyze the centre.

Rabbits weighing from 2·5–4·5 kg. were anaesthetized with Dial: (1 ml./kilo intraperitoneally). To record respiratory activity, an isolated diaphragmatic slip was attached to a recording lever after the manner of Head (1889) and carotid blood pressure was recorded. Injections were made by cannula into the jugular vein, using volumes of 1 ml. washed in by a further 1 ml. of Ringer solution at 37° C. The carbon dioxide mixture was applied during inspiration by means of a rubber balloon attached to the tracheal cannula.

As had long been known in the case of nicotine, nor-nicotine also was more toxic to the respiratory centre of the cat than to that of the rabbit, although there were wide variations due largely to the depth of anaesthesia. In a lightly anaesthetized animal with active corneal reflex, the injection of 0·5 mg. l-nor-nicotine caused a great increase in both depth and rate of respiration, the latter increasing often by 65 p.c. The respiratory centre still responded to inhalation of 6 p.c. CO_2 although the carotid blood pressure fell. After injection of 0·75 mg. of l-nicotine neither the respiratory nor the vasomotor centre responded to inhalation 6 p.c. CO_2, but the former recovered again after the injection of 1 mg. cardiazol. Larger doses, e.g., 1 mg. d-nicotine, caused a definite *retraction* of the diaphragmatic slip, an increase in both rate and depth of respiration, and a vasopressor response of 22 mm. Hg., a result which, as the protocols demonstrate, is almost quantitatively reproducible in repeated experiments. Raising the dose to 2 and 3 mg. was followed by further exaggeration of these effects.

In the lightly anaesthetized animal (vide supra) d-nicotine caused *relaxation* of the diaphragmatic slip and a depression of both rate and depth of respiration.

Both d- and l-nor-nicotines stimulated the respiratory centre but with d-nor-nicotine this was followed by a profound depression of both rate and depth of respiration from which, after a second injection, there was no recovery. d-nor-nicotine produced in the lightly anaesthetized animal the same *relaxation* of the diaphragmatic slip, as did d-nicotine.

In a similarly lightly anaesthetized animal, l-nicotine produced similar results. After artificial respiration recovery resulted, but a further dose of l-nicotine which caused increased respiratory amplitude, paralyzed the respiratory centre to the effect of cardiazol.

Quite different results were obtained with deeply anaesthetized animals. Doses of 0·5 mg. of d- and l-nor-nicotine and of d-nicotine were without effect whilst l-nicotine, after a preliminary drop in amplitude, caused an increase in this as well as in rate. With double the dose, l-nor-nicotine diminished the amplitude of respiratory movements, but not the rate, whilst l-nicotine, after an initial depression, produced the usual stimulation which a dose of 2 mg. further increased. The d-nicotine and d-nor-nicotine in doses of even 1 mg. had no effect; 2 mg. of d-nicotine stopped respiration completely. Generally speaking it may be said that the laevo-alkaloids stimulate the respiratory centre whilst the dextro varieties show mainly the depressing effect.

Action of the Alkaloids upon the Cervical Ganglion.

The classical observation in this connection is that of Langley and Dickinson (1889) who found that injection of nicotine caused dilatation of the pupil in cats and dogs, and constriction of that structure in the rabbit. They found too, that subsequent stimulation of the pre-ganglionic fibres had no effect upon the pupil, which could still, however, be influenced by stimulation of the post-ganglionic fibres. Kibjakow (1933) developed a technique for perfusing the cervical ganglion via the carotid artery, all other branches from the same being tied, and the vein from the ganglion being isolated so as to prevent injected material from gaining access to the general circulatory system. In this manner either contraction of the iris or of the nictitating membrane could be utilized to test the effect of drugs upon preganglionic nerve endings or upon post-ganglionic neurones.

In 1934, Feldberg and Vartianen modified this method to permit the use of small concentrations of l-nicotine and found that the threshold dose for causing retraction of the nictitating membrane was in the vicinity of 0·1γ. With higher concentrations, e.g., 0·5γ, paralysis of the ganglion cells followed, but after washing the ganglion out, the effect could be repeated. Complete paralysis resulted from the use of 1·0γ. Using similar technique, Hicks, v. Brücke and v. Hueber (1935) tested d-nor-nicotine and found that both iris and nictitating membrane responded to 0·5 ml. of $1·5 \times 10^4$ d-nor-nicotine. They felt that with the method of Feldberg and Vartianen only the concentration could be expressed, and that only approximately. In the present work, therefore, an attempt has been made to improve upon the technique so as to estimate if possible, the effective threshold quantity of alkaloid.

(1) Feldberg and Vartianen's method was closely followed, 1 ml. of the alkaloid solution being injected into the perfusion cannula without stopping the flow.

(2) The perfusion was interrupted by clipping the carotid; the cannula was emptied by means of a syringe and then refilled with the alkaloid solution.

(3) The ganglion was perfused by a constant pressure arrangement which permitted a change-over from the Ringer Locke reservoir to one containing a known dilution of the alkaloid in Ringer Locke. This enabled one to perfuse the ganglion for a definite period of time with a solution of known concentration, the perfusion pressure remaining constant. In all three methods the temperature at the cannula tip was controlled by means of a thermo-couple of constantan and stainless steel, and a warming resistance coil wrapped round the cannula.

Using method (1) the results of Feldberg and Vartianen were substantially verified. A concentration of 0·3γ/ml. d-nor-nicotine caused strong retraction of the nictitating membrane followed by diminishing response to stimulation of the preganglionic fibres. 1·0γ/ml. caused complete paralysis.

Using methods (2) and (3), and in particular the latter, it was found that lower concentrations of alkaloid were effective in causing stimulation. Thus, l-nicotine stimulated in concentrations of 0·01γ/ml. The method has the disadvantage of leading to a more rapid deterioration of the ganglion, due doubtless to the interruption in the perfusion and consequent effect of anoxia. The rate of perfusion varied from experiment to experiment, and decreased during the course of each.

All four alkaloids caused stimulation of the ganglion, but d-nor-nicotine only in large doses, and then at times paralysis would swiftly result without previous stimulation being recorded. With *very low* concentrations, all four alkaloids caused augmentation of the response to stimulation of the pre-ganglionic fibres. As already stated, l-nicotine by this method was effective in one-tenth of the concentration found by Feldberg and Vartianen. When the dose reached 0·5–0·8γ/ml., contraction of the nictitating membrane was followed by diminution in response to preganglionic stimulation, and by complete paralysis after 1·0γ/ml. Reversibility of this latter effect depended on perfusion rate and duration of perfusion. Thus a recently prepared ganglion perfused at a rate of 1 drop in 15 seconds will rapidly recover from the paralytic effect of 1γ/ml. of l-nicotine. In this instance stimulation resulted within 30 seconds of perfusion with the alkaloid, the membrane was completely relaxed 3·5 minutes after washing had ceased, paralysis persisted for 14 minutes and the ganglion recovered in 27 minutes, perfusion rate being 1 drop/16 sec.

d-nicotine was less active than l-nicotine, 1γ/ml. having no effect. In one experiment, 2γ/ml. gave a slight stimulation with rapidly reversible paralysis, whilst 10γ/ml. caused strong stimulation followed by completely reversible paralysis. d-nor-nicotine differed in that its paralytic effect overshadowed the stimulating action, often being the sole effect produced.

With l-nor-nicotine concentrations of 1γ/ml. were without effect. 2γ/ml. caused only augmentation of response to pre-ganglionic stimulation, whilst 3·3γ/ml. stimulated slightly, and depressed pre-ganglionic response. 5γ/ml. produced a vigorous stimulation followed by very rapidly reversible depression, and 10γ/ml. acted similarly but with final paralysis which nevertheless proved readily reversible on washing out the ganglionic vessels.

SUMMARY.

Quantitative blood pressure responses in the intact animal indicate that whilst l-nicotine is some 10 times as active as d-nicotine, l-nor-nicotine and d-nor-nicotine are approximately equal in activity and about one quarter as effective as l-nicotine.

The suprarenalectomized animal responded to the drugs in the following order: l-nicotine, 4; d-nor-nicotine, 2; l-nor-nicotine, 1; d-nicotine, 0.

On the respiratory centre the laevo-alkaloids show a preponderatingly stimulating effect whilst the dextro varieties are mainly depressant[2].

On the cervical ganglion, whilst Feldberg and Vartianen's work is verified, modified technique shows that one-tenth of the dose of alkaloid used by those authors can be effective.

The stimulating dose of d-nor-nicotine is almost identical with the paralysing dose. The order of response of the group being: l-nicotine, 300; d-nor-nicotine, 200; d-nicotine, 30; l-nor-nicotine, 1.

At *very low* concentrations *all four alkaloids* caused augmentation of response to stimulation of the pre-ganglionic fibre.

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PROTOCOL 1.

Effect on blood pressure.

Cat weighing 2·2 kg.

Time.

10.00 Anaesthetized with Dial—2·4 ml.
11.30 Eviscerated.
12.15 Dose of atropin 0·5 mg.

	Drug.	Dose mg.	Rise in blood pressure (mm. Hg.).	Remarks.
2.10	l-nicotine	0·1	no effect	
2.15	l-nicotine	0·2	5	
2.25	l-nicotine	0·5	10	Respiration laboured, tendency to forced expiration
2.35	l-nicotine	1	17·5	
2.40	l-nicotine	1	17·5	Respiration ceased
2.42	l-nicotine	1	16	
2.47	d-nicotine	1	4	
2.50	d-nor-nicotine	1	7	
2.54	l-nor-nicotine	1	8	

[2] In these experimental animals the carotid sinus mechanism was intact, and research shortly to be published from this laboratory by David I. B. Kerr indicates that this mechanism may have been responsible for the respiratory responses here reported.

PROTOCOL 1.

Cat weighing 1·9 kg.

Time.				
9.30	Anaesthetized with Dial—2 ml.			
10.00	Eviscerated.			
10.45	Atropin injected—1 mg.			
	Drug.	Dose mg.	Rise in blood pressure (mm. Hg.).	Remarks.
11.50	l-nicotine	0·5	59	No effect on respiration
12.00	l-nor-nicotine	0·5	14	Increased respiration
12.09	l-nor-nicotine	0·5	14	Increased respiration
12.19	l-nicotine	0·5	53	Increased respiration, muscular tremors
12.25				Artificial respiration
12.30	d-nicotine	0·5	6	Started
12.35	l-nicotine	0·5	40	
12.40	d-nicotine	0·5	6	
2.15				Respiratory efforts, muscular tremors ceased
2.30	l-nicotine	1	46	
2.40	l-nor-nicotine	1	11	
2.50	l-nicotine	1	42	
2.51	d-nicotine		6	
3.04	l-nicotine	1	35	
3.10	d-nor-nicotine		6	
3.20	l-nicotine	1	30	
3.28	d-nor-nicotine	2	6	
3.30	l-nicotine	1	10	Suprarenals removed
to	d-nicotine	1	7	
3.54	l-nor-nicotine	1	8	
	d-nor-nicotine	1	6	

Cat weighing 4·4 kg.

Time.				
12.14	Suprarenals removed.			
12.55	Atropin injected—1 mg.			
	Drug.	Dose mg.	Rise in blood pressure (mm. Hg.).	Remarks.
2.20	l-nicotine	0·25	20·5	Increased respiration
2.51	d-nicotine	0·25	5·5	No increase in respiration
3.10	d-nor-nicotine	0·25	12	
3.16	l-nor-nicotine	0·25	6	
4.25	d-nicotine	1	10·5	
4.30	d-nor-nicotine	1	14	
4.40	l-nor-nicotine	1	8	Forced respiration and muscle twitches
4.50	l-nicotine		22·5	Eviscerated
5.20				Animal died

PROTOCOL 2.

Rabbit weighing 2·4 kg.

Dial 2·4 ml., ether.
Blood pressure at beginning of experiment 103 mm. Hg.

Time.	Drug.	Dose mg.	Blood pressure mm. Hg.	Respiration rate No./min.	Remarks.
11.30	l-nicotine	0·5	incr. 10	16–28	Increased depth of respiration
11.50	6 p.c. CO_2		decr. 9	14–17	Increased depth of respiration
12.00	6 p.c. CO_2		decr. 8	14–20	Increased depth of respiration
12.20	6 p.c. CO_2		decr. 6	14–21	
2.03	6 p.c. CO_2		no change	14–17	
2.15	6 p.c. CO_2		decr. 7	14–15	
2.40	l-nicotine	0·75	decr. 7 and incr. 3	13–15	
2.42	6 p.c. CO_2		no change	16–17	No increase in amplitude of respiration
3.15	6 p.c. CO_2		decr. 9	12–13	Cardiazol 1 mg.
3.20	l-nicotine	1	incr. 16	14–23	Contraction of diaphragm and increase in depth of respiration
3.40	l-nicotine	2	incr. 22	14–19	Contraction of diaphragm and increased depth of respiration
3.45	d-nicotine	1	decr. 2	12–17	
3.53	l-nor-nicotine	1	incr. 2	no change	
4.05	d-nicotine	1	incr. 2	no change	
4.20	l-nicotine	3	incr. 30	14–26	
4.30	l-nicotine	1	incr. 10	12–22	Cardiazol 1 mg.
4.45	d-nicotine	2	decr. 6·5	16–14	Relaxation of diaphragm
4 50	d-nicotine	2	decr. 6·5	16–15	Slight depression of respiration and decrease in depth
5.17	l-nicotine	1	incr. 14	14–26	Slight contraction of diaphragm
5.40	l-nicotine	2	incr. 4	14–18	
5.42	l-nor-nicotine	2	decr. 4	14–18	
5.50	d-nor-nicotine	2	decr. 6·5	14–9	Respiration ceased after second dose

PROTOCOL 2 (continued).

Rabbit weighing 2·3 kg.

Time. 9.50 — 2·5 ml. Dial, ether.
Normal blood pressure—105 mm. Hg.
Light anaesthesia.

	Drug.	Dose mg.	Blood pressure mm. Hg.	Respiration rate No./min.	Remarks.
11.55	d-nicotine	0·5	no effect	24	
12.02	l-nicotine	0·5	incr. 22·5	21–23	Contraction of diaphragmatic slip, increase in depth of respiration
12.07	d-nor-nicotine	0·5	incr. 5	20–21	Slow relaxation of diaphragm and depression of respiration. Recovery complete in 20 minutes
12.31	l-nor-nicotine	0·5	incr. 9	16–12	Depression of amplitude and increasing showing of rate. Final cessation of respiration after 4 minutes
12.50	l-nicotine	0·5	decr. 15	18–16	No contraction of diaphragmatic slip, but increase in amplitude, followed by slowing of rate, and cessation. Cardiazol was not effective

PROTOCOL 2.

Rabbit weighing 2·5 kg.

Normal blood pressure 105 mm. Hg.

Time. 1.10 — Dial 3 ml., no ether.

	Drug.	Dose mg.	Blood pressure mm. Hg.	Respiration rate No./min.	Remarks.
2.00	Cardiazol	1		16	
2.07	l-nicotine	0·5			No effect
2.10	d-nor-nicotine	0·5			No effect
2.16	d-nicotine	0·5	decr. 4		No effect on respiration
2.18	l-nicotine	0·5	decr. 14	14–20	Slight contraction of diaphragmatic slip
2.20	l-nor-nicotine	1·0	incr. 5	18–19	Slow decrease in amplitude
2.43	l-nicotine	1	decr. 14	18	Rapid decrease in amplitude followed by increase
2.49	d-nor-nicotine	1	incr. 2	17–16	No effect on amplitude
2.54	d-nicotine	1	no effect	16 unaltered	Slight decrease in depth
2.59	l-nicotine	2	decr. 16	15–23	Decrease in depth followed by marked increase
5.09	d-nicotine	2	no effect	13–10	Gradual decrease in amplitude. Animal stopped breathing, cardiazol ineffective

PROTOCOL 3.

Perfusion of superior cervical ganglion.

Expt.	Drug.	Dose.	Technique.	Augmentation of effect of pre-ganglionic stimulation.	Retraction nictitating membrane.	Effect on ganglion.
9	l-nor-nicotine	0·5	1st	none	positive	paralysis in 3 mins.
		0·33		none	positive	paralysis and recovery in 20 mins.
8		0·1		positive	none	none
		0·2		none	none	slow paralysis, no complete recovery
9		0·1		none	none	none
		0·2		positive	none	none
		0·5		none	none	slow paralysis
1	l-nicotine	0·3	1st	positive	strong	none
		0·4		positive	strong	inhibition of effects of stimulation recovers in 8 mins.
		0·5		none	positive	depression
		0·8		none	positive	paralysis without complete recovery
		0·1		none	slight	complete paralysis for 14 mins., effect of stimulus almost normal in 7·5 mins.
2	l-nicotine	1·0	2nd	none	strong	complete paralysis for 14 mins., effect of stimulus almost normal in 7·5 mins
		0·01	2nd	positive	slight	none
		0·01	2nd	positive	slight	none
1	d-nor-nicotine	0·6	1st	positive	none	none
		0·2		none	none	slow recovery on washing out
		0·6		none	none	slow paralysis, no recovery
2	d-nor-nicotine	0·001	2nd	none	none	none
		0·005		no effect	no effect	no effect
		0·01		no effect	no effect	no effect
		0·5		none	none	depression of stimulation pre-ganglionic
		0·06		none	none	depression of stimulation pre-ganglionic
		0·07			slight	slight depression but rapid recovery
		0·1			slight	slow paralysis, no recovery in 35 mins.

PROTOCOL 3 (continued).

Expt.	Drug.	Dose.	Technique.	Augmentation of effect of pre-ganglionic stimulation.	Retraction nictitating membrane.	Effect on ganglion.
3	d-nor-nicotine	0·1	3rd	none	slight	no effect
		0·11		positive	none	none
		0·125		positive		
		0·14		none	none	depression of pre-ganglionic stimulus, rapid recovery
		0·2		none	none	slow paralysis, no recovery
	second ganglion					
		0·2			slight	slow paralysis
		0·25			slight	paralysis in 8 mins., recovery in 14 mins.
4	d-nor-nicotine	0·2		none	none	paralysis in 30 secs., recovery in 19 mins.
5						
6	inconclusive					
7						
3	l-nicctine	0·01	3rd	positive	small and definite	none
4	l-nicotine	0·2	3rd	positive	definite	none
5	l-nicotine	0·1	3rd	none	none	none
	second ganglion	1·0		none	slight	paralysis
		0·1		positive	strong	none
		0·05		positive	none	none
		0·1			slight	depression of effect of pre-ganglionic stimulation
		0·125			definite	as with 0·1
		0·2			definite	marked depression of pre-ganglionic stimulation
		0·1			strong	paralysis, recovery on washing out
1	l-nor-nicotine	1	3rd	positive	none	none
		5			strong	paralysis in 15 mins., recovery in 3 mins.
		2		positive	none	none
		3·3			small	depression
		10			strong	complete paralysis, no recovery but stimulation of post-ganglionic fibres effective
2		1	very slow perfusion	none	none	paralysis
3		1		positive	none	none
		0·2		none	none	depression
		10		none	strong	paralysis and recovery

PROTOCOL 3 (continued).

Expt.	Drug.	Dose.	Technique.	Augmentation of effect of pre-ganglionic stimulation.	Retraction nictitating membrane.	Effect on ganglion.
4		0·5		none	none	none
		1·0		positive	none	none
		10		none	strong	paralysis and recovery
1	d-nicotine	10		none	strong	depression and rapid recovery
1	d-nicotine	50	3rd	none	strong	paralysis
		10		none	definite	depression
2	d-nicotine	20		none	slight	depression
		38		none	definite	depression
		10		none	strong	paralysis

End